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Simultaneous high performance liquid chromatographic determination of procainamide, N-acetylprocainamide, disopyramide, mono-N-dealkyldisopyramide, quinidine, and propranolol in serum

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SIMULTANEOUS HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PROCAINAMIDE, N-ACETYLPROCAINAMIDE, DISOPYRAMIDE, MONO-N-DEALKYLDISOPYRAMIDE, QUINIDINE, AND PROPRANOLOL IN SERUM

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Simultaneous high performance liquid chromatographic determination of procainamide, N-acetylprocainamide, disopyramide, mono-N-dealkyldisopyramide, quinidine, and propranolol in serum. I, James F. Wesley, hereby grant permission to the Wallace Memorial Library of R.I.T., to reproduce the document titled above in whole or in part. Any reproduction will not be for commercial use or profit.

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ABSTRACT

We describe a method for the simultaneous high performance liquid chromatographic determination of several antiarrhythmic drugs and some of their metabolites after extraction from 2.5 ml of spiked pooled sera. The extracts were applied to a C8 reversed phase column. Nine compounds of interest were resolved within the 30 minute run. An initial mobile phase of 80% phosphate (25 mmol/L, pH 3.5), 20 % organic (acetonitrile: methanol, 2:3) was maintained for 2 min at which time a linear gradient was used to change the mobile phase to 30% phosphate, 70% organic at 20 min after injection. This composition was maintained for an additional 5 min. Absorbance at 212 nm was used for detection. Peak area ratios of drug to internal standard (N-propionylprocainamide) were used for quantitation. The relative standard deviations (and mean solute concentrations) of daily duplicate determinations for 15 days are: procainamide (PA), 5.1% (5.9 mg/L); N-acetylprocainamide (NAPA), 9.3% (6.0 mg/L); mono-N-dealkyldisopyramide (NDAD), 3.7% (4.1 mg/L); disopyramide (DISOP), 4.3% (4.0 mg/L); quinidine (QUIN), 4.5% (6.5 mg/L); propranolol (PROPL), 5.1% (97 µg/L); and dihydroquinidine (DIHYQ), 9.3% (0.69 mg/L). A propranolol metabolite, 4-hydroxypropranolol (4-OHP), was resolved but not quantitated.

INTRODUCTION

Quinidine, procainamide, disopyramide, and propranolol are drugs that are prescribed for their cardiac antiarrhythmic qualities. The determination of the blood serum concentration of each drug may be useful to the physician in an effort to improve the effectivenes of therapy.

Quinidine is a myocardial depressant (as are procainamide and disopyramide) that may cause serious side effects at serum concentrations that are only ten percent higher than the therapeutic range. These include life threatening arrhythmias, serious hypotension, and hypersensitivity reactions (1).

Procainamide usage in certain patients, characterized as "slow acetylators", may produce a potentially fatal syndrome similar to Lupus Erythematosus. This condition has been related to the rate of conversion of procainamide to its pharmacologically active, acetylated metabolite, N-acetylprocainamide. Nausea and weakness may occur at procainamide serum levels fifteen percent higher than the therapeutic range. Serum concentrations of N-acetylprocainamide are not known when these toxic symptoms appear (1).

Disopyramide may cause worsening arrhythmia and electrocardiogram changes with high doses and increased serum concentrations. Mono-N-dealkyldisopyramide is a major metabolite whose pharmacoactivity is still undetermined (1).

Propranolol is a β-adrenergic blocking agent which affects the cardiac action of the catecholamines. There is poor correlation of drug dosage with blood concentration that is believed to be due to large variations in the rate of metabolism (1). Serum levels are usually requested to ensure that a minimum therapeutic concentration has been achieved and/or if the patient is in compliance with therapy. Side effects at high levels may rarely include congestive heart failure and pulmonary edema. One of the fifteen identified metabolites, 4-hydroxypropranolol, is believed to possess antiarrhythmic activity (2).

Our objectives in developing a high performance liquid chromatographic method for the quantitation of procainamide, N-acetylprocainamide,

disopyramide, mono-N-dealkyldisopyramide. quinidine, and propranolol were threefold. First, we sought to develop an assay for these drugs that would be free from major interferences that are present in the fluorometric methods for quinidine (3) and propranolol (4) and the colorimetric method for procainamide (5). Secondly, we wanted a means of detecting and quantitating serum levels of the major drug metabolites, whether or not they are reported to have pharmacologic activity, to aid in drug dosage regimens. Lastly, we needed a method that could be used for the simultaneous determination of as many of the antiarrhythmic drugs and metabolites as possible. In this way, we could best utilize a microprocessor controlled liquid chromatograph with an autosampling system that could run unattended and perform an analysis on an extracted specimen regardless of which drug or combination of drugs was present in the sample.

Miller and Tacker have reviewed previous HPLC methods for multi-component serum analysis (6). To our knowledge, this is the first report of a method that can be used for the simultaneous measurement of several antiarrhythmic drugs and metabolites in serum.

EXPERIMENTAL

APPARATUS

A Hewlett-Packard Model 1084B high performance liquid chromatograph was used in this study. It is a microprocessor controlled system in which keyboard entries are used to control several parameters; including a linear, variable by time, elution gradient; mobile phase flow rate; column temperature; and detector wavelength selection. The microprocessor also integrates peak areas by continuously monitoring the slope of the chromatogram. The sample injector is a variable volume syringe that can be operator adjusted to deliver 10 to 200 µL. An automatic sampling system allows up to 60 samples to be run without further operator intervention. The variable wavelength detector is a single cell, dual wavelength type with an operating range of 190 nm to 600 nm.

An Altex Ultrasphere Octyl C8 (25 cm x 4.6 mm i.d.) column (Rainen Instrument Co., Woburn, MA 01801) was used for the separations. A Brownlee RP8 guard column (Rainen Instrument Co., Woburn, MA 01801) was used to prolong the analytical column lifetime.

Clin-Elute CE-1003 columns (Analytichem International, Inc., Lawndale, CA 90260) were used to extract the drugs and internal standard from serum.

Silica Gel 60 (0.2 mm thickness) pre-coated thin layer chromtography sheets (EM Reagents, American Scientific Products, McGaw Park, IL 60085) were used as the TLC stationary phase.

REAGENTS

All aqueous solutions were prepared with deionized water that had been passed through a 0.22 µ filter. Acetonitrile and methanol were HPLC grade (Fisher Scientific Co., Pittsburgh, PA) and used without further purification. Reagent grade H_3PO_4, $NaH_2PO_4 \cdot H_2O$, concentrated NH_4OH, and ethyl acetate (Fisher Scientific Co.) were used as received.

The drugs, metabolites, and internal standard, used to prepare stock methanol solutions, were not purified further. Quinine, N-acetyl procainamide (both from Sigma Chemical Co., St. Louis, MO 63178), and N-propionylprocainamide (Pierce Chemical Co., Rockford, IL) were

purchased. The following were gifts supplied by the respective manufacturers: procainamide·HCl (E.R. Squibb & Sons, Princeton, NJ 08540); disopyramide phosphate and mono-N-dealkyldisopyramide (G.D. Searle and Co., Chicage, IL 60680); propranolol·HCl and 4-hydroxypropranolol (Ayerst Laboratories, Inc., New York, NY); quinidine·SO_4 (Eli Lilly and Co., Indianapolis, IN 46206); and dihydroquinidine (A.H. Robbins, Co., Richmond, VA 23220).

Stock solutions were prepared in calibrated conical centrifuge tubes by dissolving each substance in an appropriate amount of HPLC grade methanol (approximately 5 mL) to obtain a concentration of 1.0 g of free drug/L.

Working methanol stock solutions were prepared for N-propionylprocainamide and propranolol of 0.2 g/L, respectively, by appropriate dilution of the concentrated stocks. To preserve the integrity of the 4-hydroxypropranolol, which is unstable for long periods in methanol, 60 µL of stock solution was transferred to several test tubes, the methanol evaporated under N_2 at 50° C, and the residue was stored stoppered at room temperature (60 µg per tube).

PROCEDURE

The aliquots of frozen pooled sera (standards, control, and blank are thawed at room temperature and vortexed for 10s to ensure homogeniety.

Pipet 200 µL of 0.1 mol/L HCl into labeled 16 x 150 mm glass collection tubes that are then positioned below a corresponding Clin-Elute column. The pH of the column is adjusted by adding 200 µL of 1 mol/L Na_2CO_3. This is followed by the addition of internal standard, 50 µL of N-propionylprocainamide (0.2 g/L in methanol). Pipette 2.5 mL of serum onto the column and wait 3 min for equilibration. Three additions of 4 mL each of the extraction solvent (methylene chloride:hexane:isoamyl alcohol, 49:49:2, v:v) follow. Wait for the dripping to stop before adding the next portion. Vortex the collection tubes for 45s and centrifuge at 2000 rpm for 10 min. Aspirate and discard the organic supernatant. Add 300 µL of methanol to the remaining aqueous phase in the collection tube, vortex for 5s, and evaporate to dryness at 50° C under a gentle stream of N_2. Dissolve the residue in 150 µL of the initial mobile phase (described below) and inject 75 µL into the liquid

chromatograph.

CHROMATOGRAPHIC CONDITIONS

The mobile phase was in two reservoirs: one contained 25 mmol/L phosphate, pH 3.5, and the other, a mixture of acetonitrile and methanol (2:3). The phosphate was prepared by mixing 25 mmol/L solutions of H_3PO_4 and NaH_2PO_4 until a pH of 3.5 was achieved. This aqueous solution was then passed through a 1.2 μ Millipore filter under vacuum which also effectively de-gassed the solution. The organic mixture was de-gassed by heat and vacuum in the chromatograph reservoir.

The elution was performed at 30^{o} C with a flow rate of 1.0 mL/min. The initial mobile phase composition was 80% phosphate and 20% organic. At 2 min after the injection of sample, a linear gradient was started that reached 30% aqueous and 70% organic after 20 min into the run. This mixture was maintained for another 5 min when the run was terminated. The mobile phase was returned to its initial composition and allowed to equilibrate with the column for 5 min before another sample was chromatographed. Total run time was therefore 30 min.

The variable wavelength detector, with a 12 μL, 10 mm flow cell, was set at 212 nm for sample monitoring. The reference wavelength was 430 nm. A chromatogram of all assay components is presented in Figure 1.

LINEARITY

The linearity of detector response, as a function of the concentration of each component of interest, was confirmed by comparison of the peak areas of two-fold serially diluted mixtures in mobile phase. Table 1 lists the range of each substance for which the area did not deviate more than 10% from the expected dilution factor. The serum concentrations that would correspond to the measured linearity range by use of our extraction procedure, assuming 100% recovery, are also shown. The accepted therapeutic ranges are included for comparative purposes.

The 4-hydroxypropranolol proved to be unstable in methanol (i.e. stock solutions) and in mobile phase. The stability of a fresh solution was sufficient to determine the chromatographic retention time, but it was not adequate for quantification. This propranolol metabolite is reported to have amphoteric extraction characteristics (7), and a serum concentration of 5-40 μg/L (8). It was concluded from this

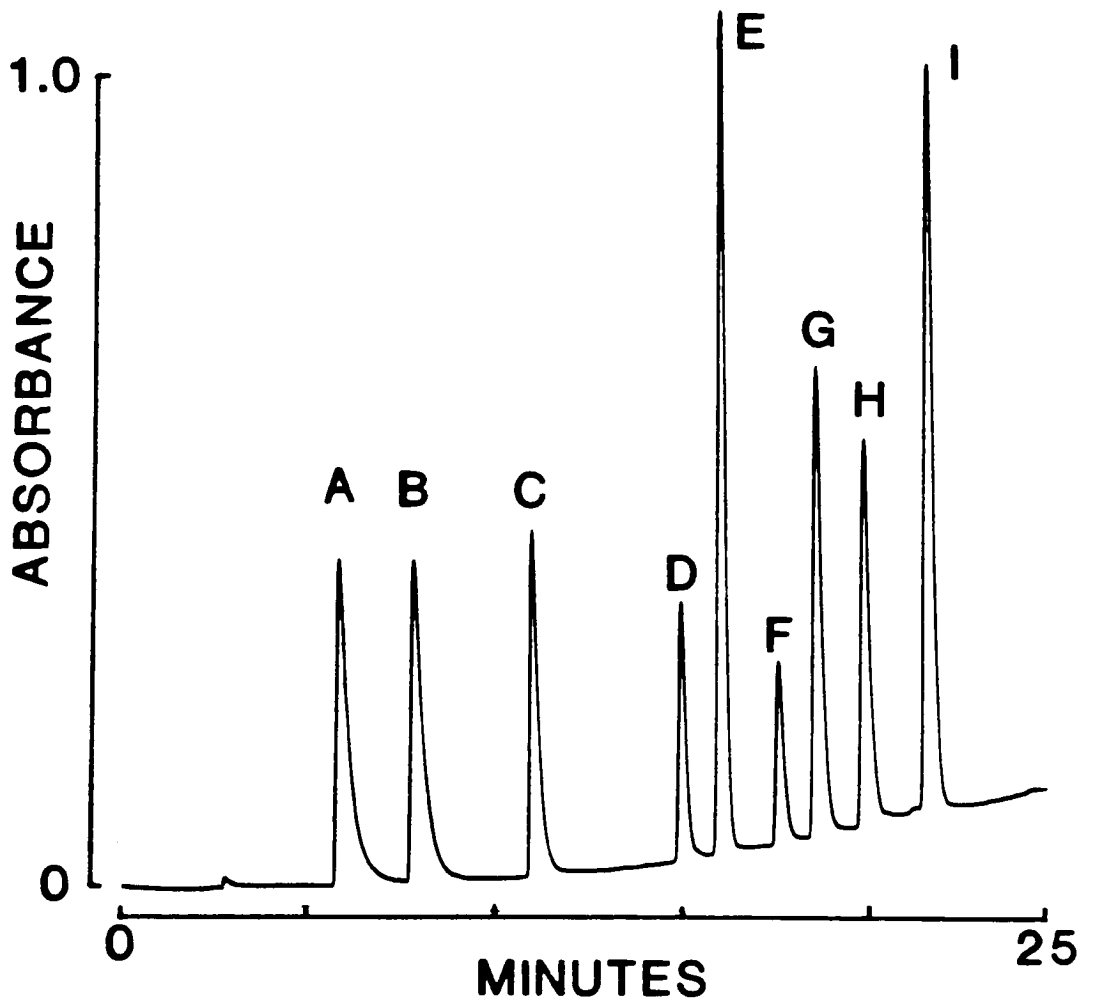

Fig. 1. Chromatogram of antiarrhythmic drugs, metabolites, and internal standard. The components (1.5 μg each) are identified by their respective retention times, are: A, procainamide (5.68 min); B, N-acetylprocainamide (7.64 min); C, N-propionylprocainamide (10.88 min); D, mono-N-dealkyldisopyramide (14.90 min); E, 4-hydroxypropranolol (15.94 min); F, disopyramide (17.51 min); G, quinidine (18.51 min); H, dihydroquinidine (19.79 min); and I, propranolol (21.49 min).

TABLE 1. DETECTOR LINEARITY AT 212 nm.

Substance	Amount Injected (µg)	Equivalent Serum Concentration[a] (mg/L)	Therapeutic Serum Concentration[c] (mg/L)
Procainamide	0.27 - 17.5	0.22 - 14.0	4 - 8
NAPA	0.14 - 17.5	0.11 - 14.0	4 - 8
NPP	0.14 - 17.5	b	-
NDAD	0.03 - 17.5	0.03 - 14.0	?
Disopyramide	0.07 - 17.5	0.05 - 14.0	2 - 4
Quinidine	0.14 - 17.5	0.11 - 14.0	3 - 6
Propranolol	0.005 - 0.33	3 - 210[d]	20 - 100[d]

[a] Assumes 100 percent recovery in reported extraction procedure (see Table 3 for actual recoveries).

[b] Internal standard.

[c] From reference (1).

[d] Concentration µg/L.

information that 4-hydroxypropranolol was an inappropriate compound to include in this assay.

Linearity of the overall assay procedure was determined through the use of calibration curves. These were prepared from spiked sera with the use of N-propionylprocainamide as the internal standard. The peak area ratio of drug /internal standard (ordinate) was plotted against the concentration of drug in the serum spike (abscissa). The high standard is approximately twice the high therapeutic serum concentration in each case. Serial dilutions of the high standard to concentrations of 1/3 and 1/9, using the same serum pool but without drug or metabolite as the diluent, were prepared to cover the full range of clinical interest. Standard curves for all drugs and metabolites are shown in Figures 2-8. Linear regression analysis data of the standard curves are summarized in Table 2. The small values for the Y-intercept indicates that no endogenous interferences are present. This was confirmed by extraction and elution of blank sera.

Absolute recovery was studied by the comparison of peak areas from stock solution residues, that had been reconsituted in mobile phase, with the peak areas from extracts of a spiked serum pool. Extraction efficiencies for several serum concentrations of each drug are summarized in Table 3. The internal standard (N-propionylprocainamide) had an absolute recovery of 82% (SD $\pm$ 4% n=12) when 50 µL of a 0.2 g/L methanol solution was added to the Clin-Elute column as described in the procedure. Although the absolute recoveries of N-acetylprocainamide and propranolol are less than 100%, they are also independent of concentration and therefore should be adequate for clinical use.

PRECISION

The reproducability of the injection system of the chromatograph was determined by injecting pure drug standards (n=10). The variation in retention times for any analyte was always less than 0.1 min. The relative standard deviation of each chromatographic peak area was less than 4% for all of the drugs except for the 4-hydroxypropranolol. When peak area of each drug was divided by the area of the internal standard, relative standard deviations improved to less than 1%.

Precision studies of drug quantification were conducted with two pools of spiked sera, one for within-run variation and one for run-to-

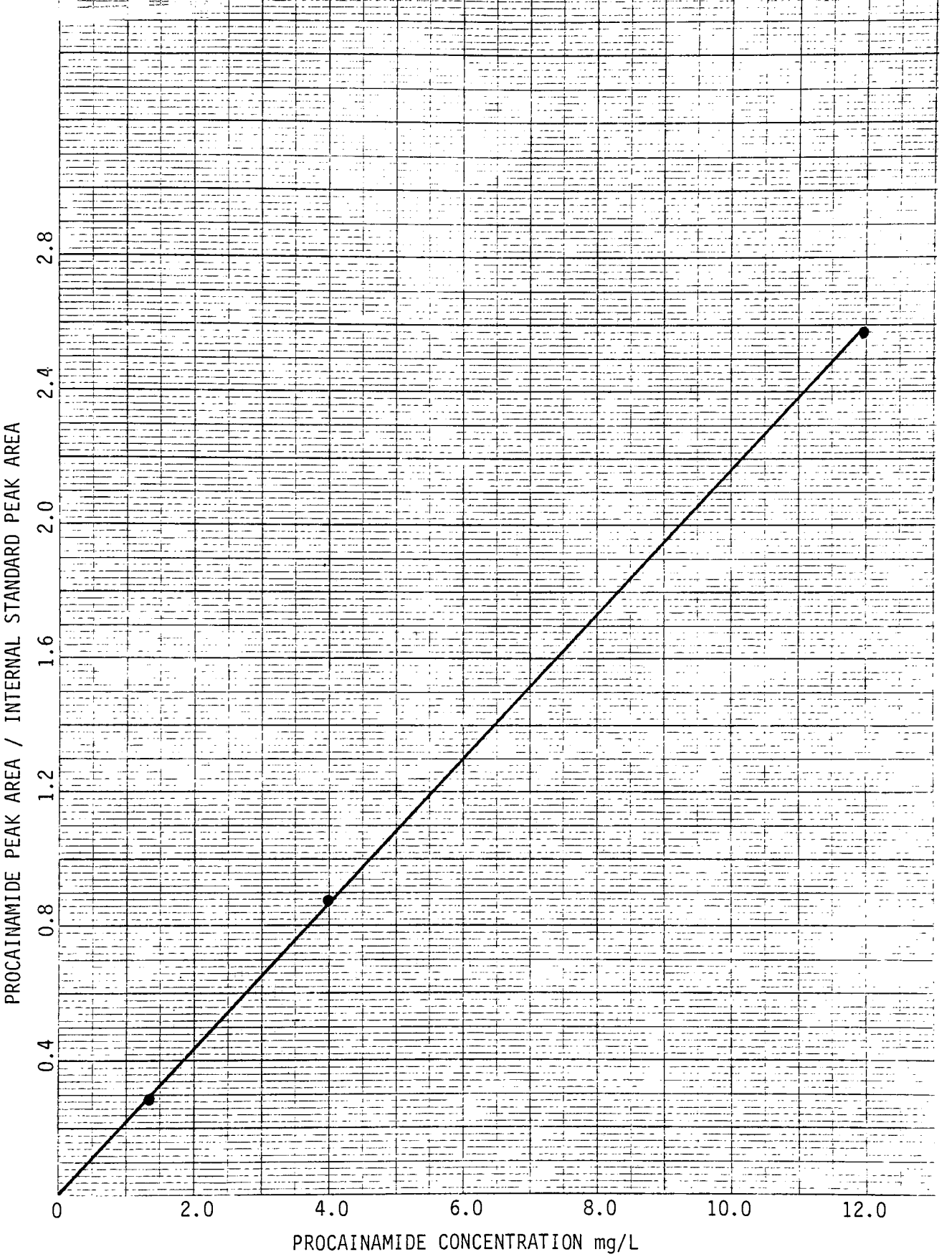

Fig. 2. Procainamide calibration curve from spiked serum extracts. Comparison of peak area drug/peak area internal standard with drug concentration in mg/L. Each point represents an average of three determinations. Standards are: 12.0 mg/L, 4.0 mg/L, and 1.3 mg/L.

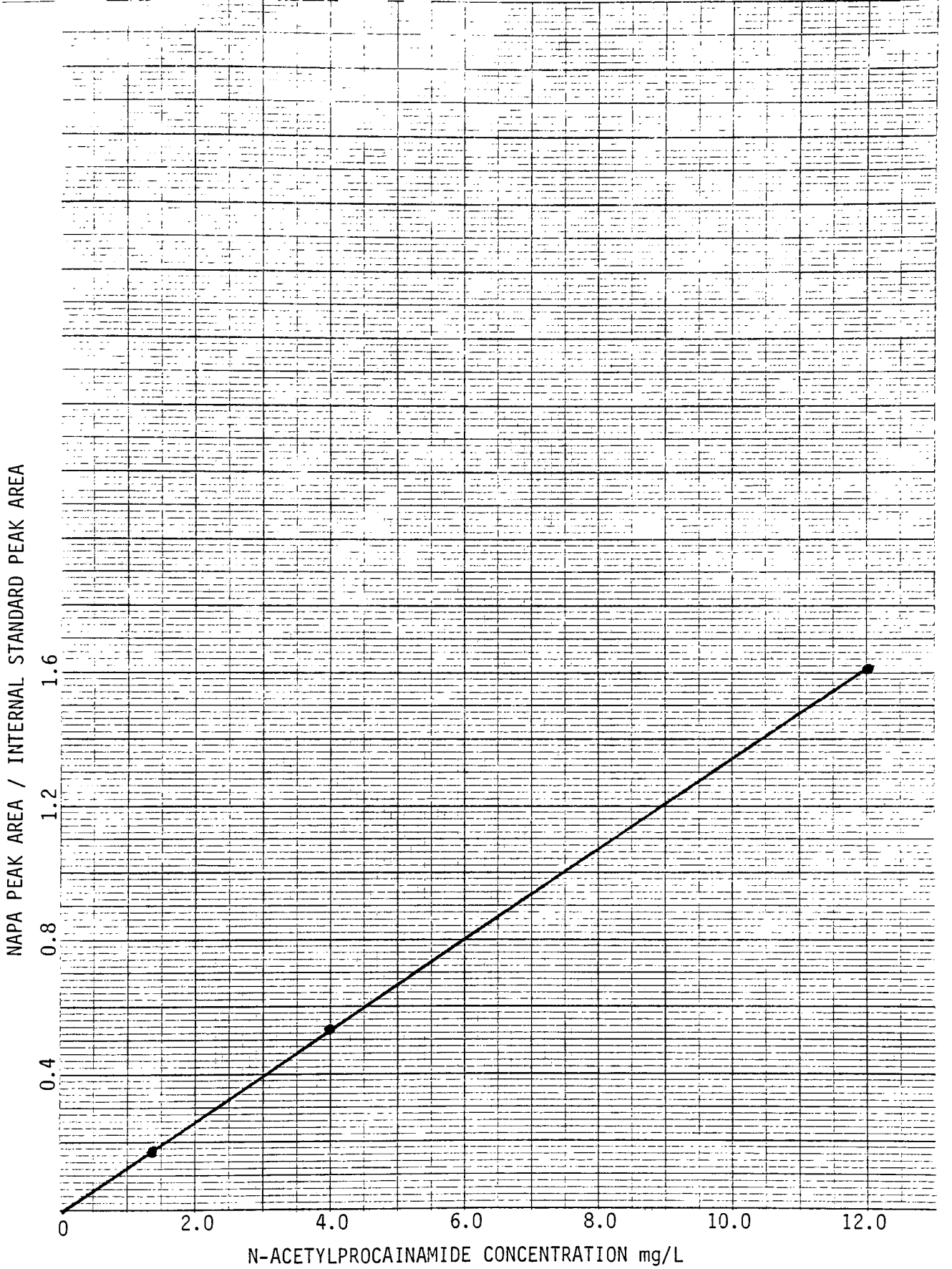

Fig. 3. N-acetylprocainamide calibration curve from spiked serum extracts. Comparison of peak area drug/peak area internal standard with drug concentration in mg/L. Each point represents an average of three determinations. Standards are: 12.0 mg/L, 4.0 mg/L, and 1.3 mg/L.

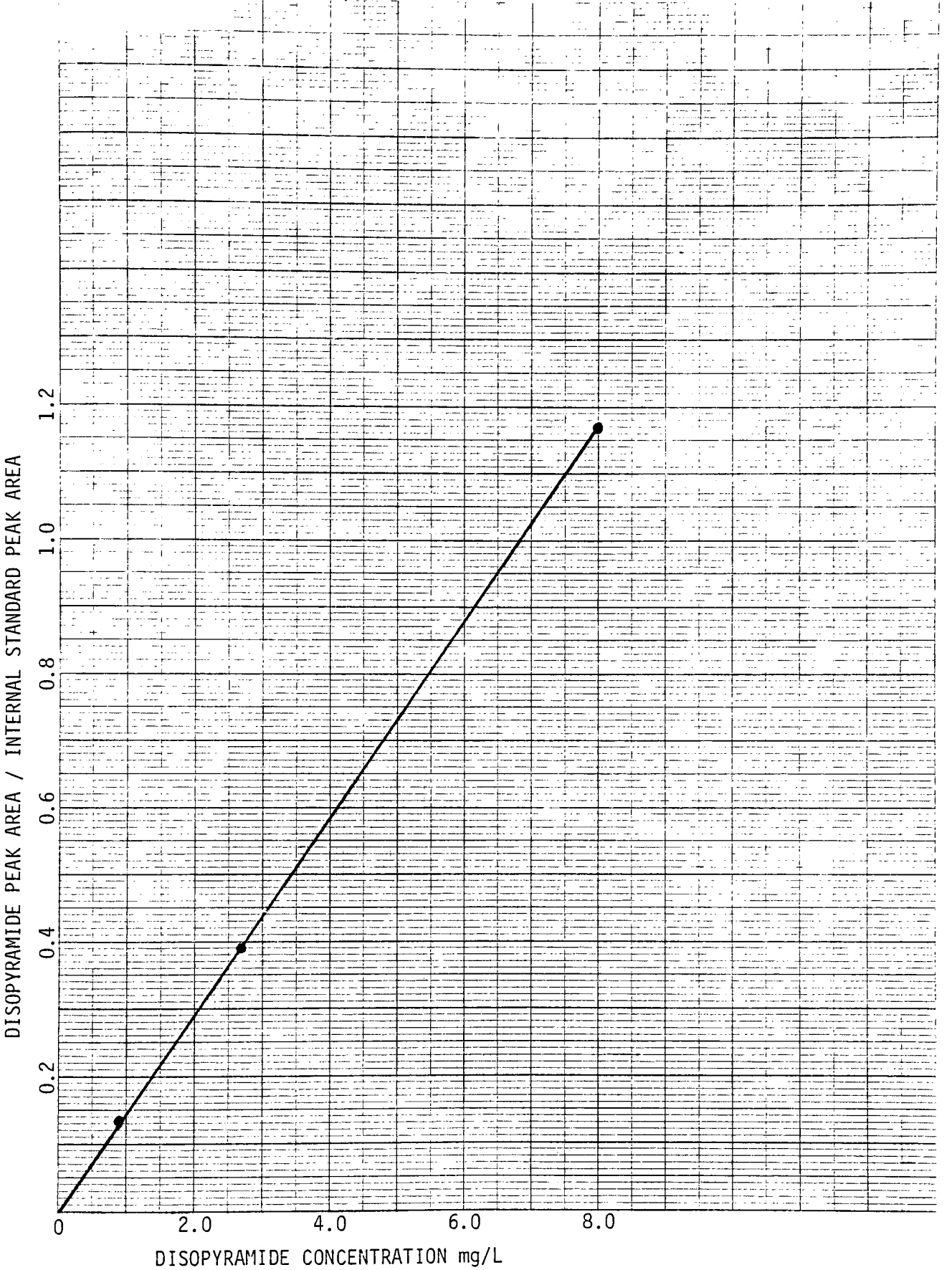

Fig. 4. Disopyramide calibration curve from spiked serum extracts. Comparison of peak area drug/peak area internal standard with drug concentration in mg/L. Each point represents an average of three determinations. Standards are: 8.0 mg/L, 2.7 mg/L, and 0.9 mg/L.

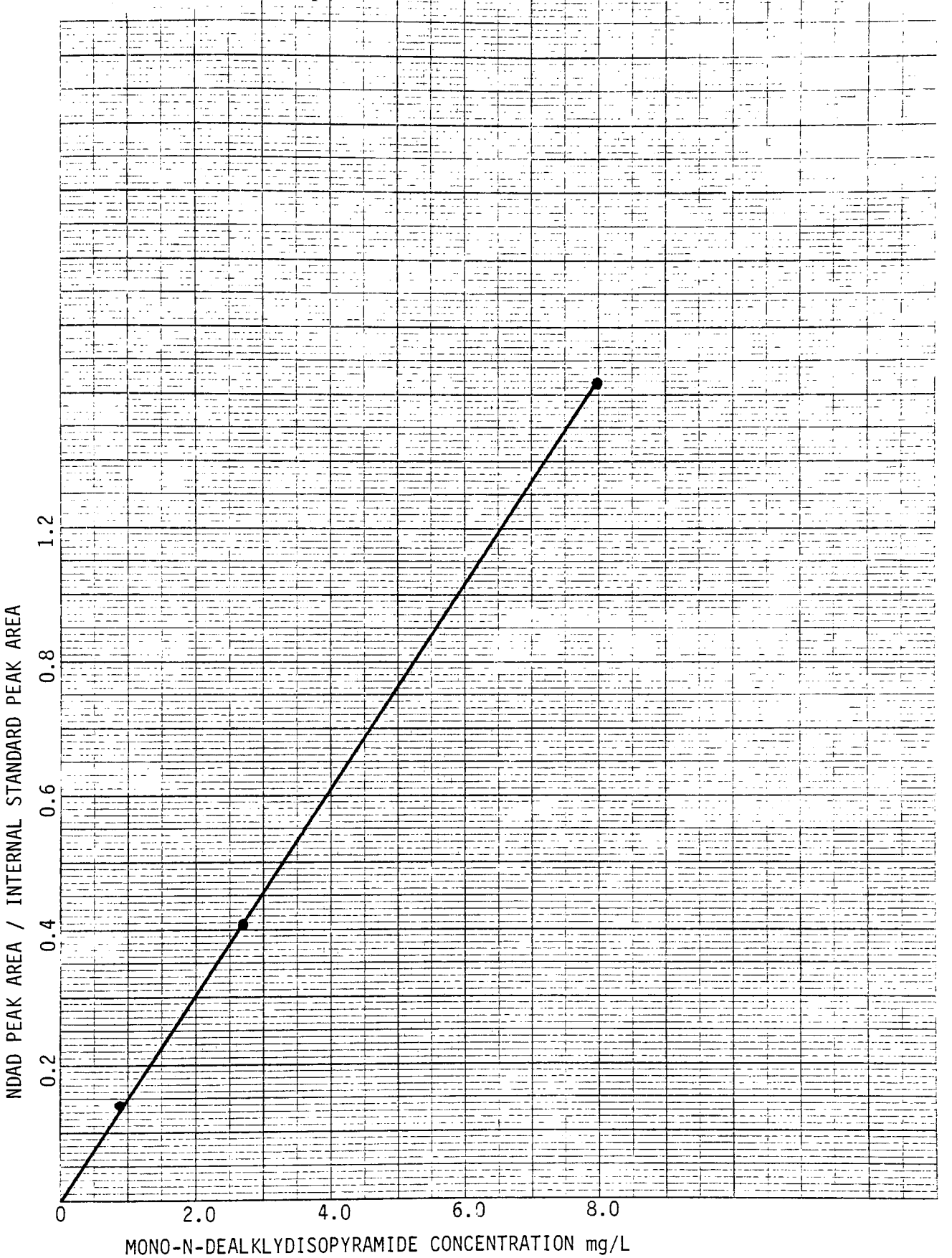

Fig. 5. Mono-N-dealkyldisopyramide calibration curve from spiked serum extracts. Comparison of peak area drug/peak area internal standard with drug concentration in mg/L. Each point represents an average of three determenations. Standards are: 8.0 mg/L, 2.7 mg/L, and 0.9 mg/L.

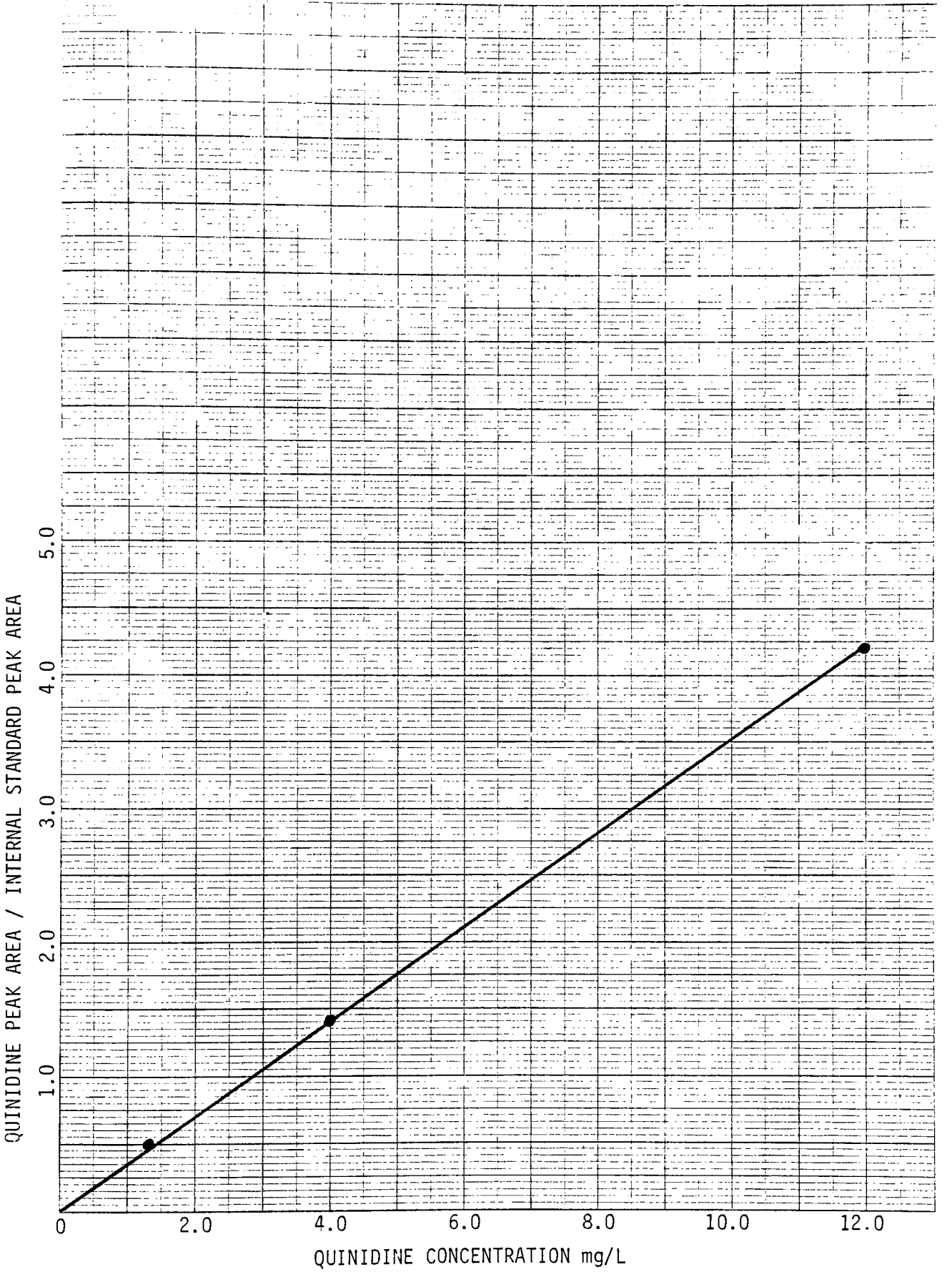

Fig. 6. Quinidine calibration curve from spiked serum extracts. Comparison of peak area drug/peak area internal standard with drug concentration in mg/L. Each point represents an average of three determinations. Standards are: 12.0 mg/L, 4.0 mg/L, and 1.3 mg/L.

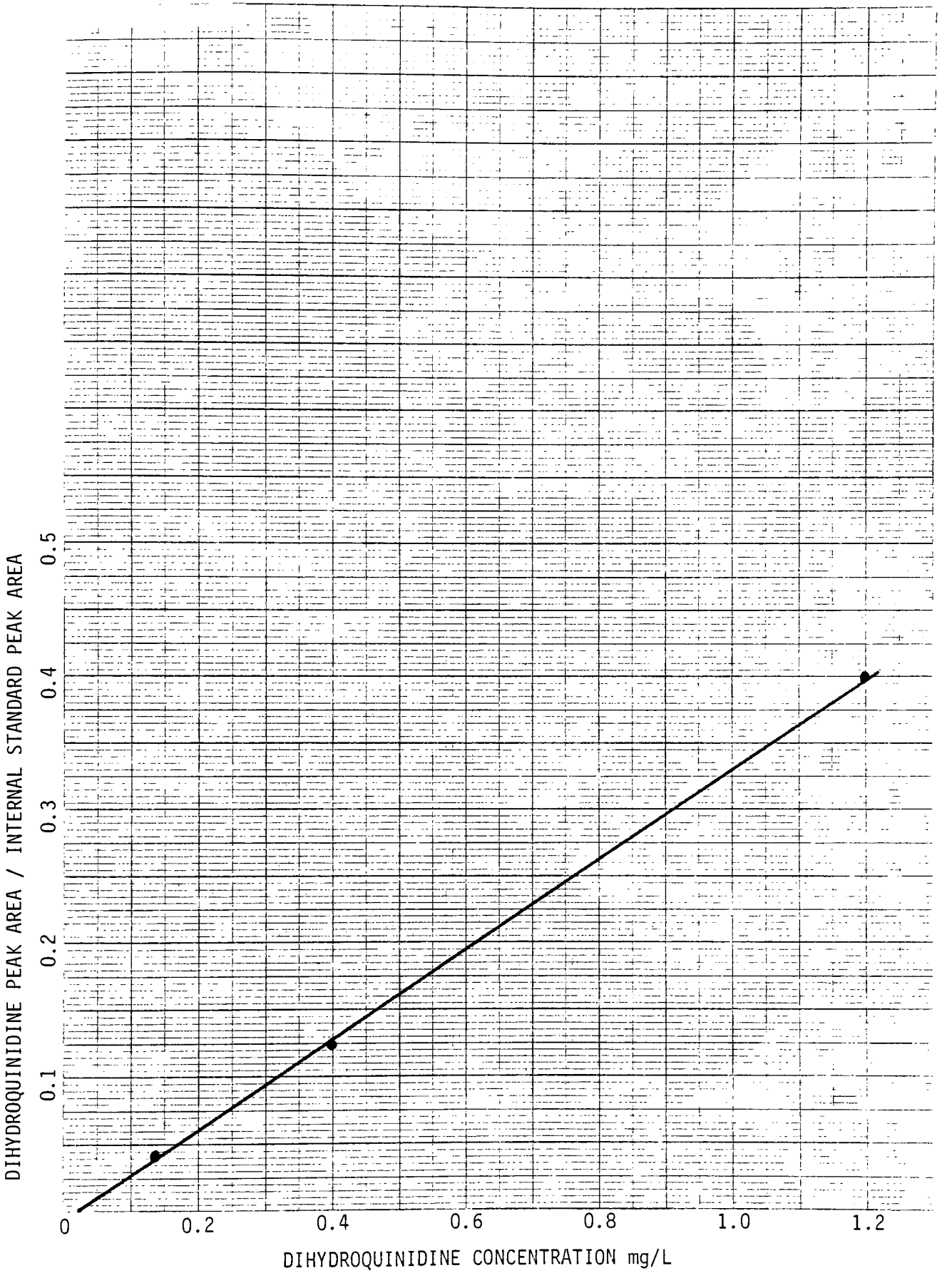

Fig. 7. Dihydroquinidine calibration curve from spiked serum extracts. Comparison of peak area drug/peak area internal standard with drug concentration in mg/L. Each point represents an average of three determinations. Standards are: 1.2 mg/L, 0.4 mg/L, and 0.13 mg/L.

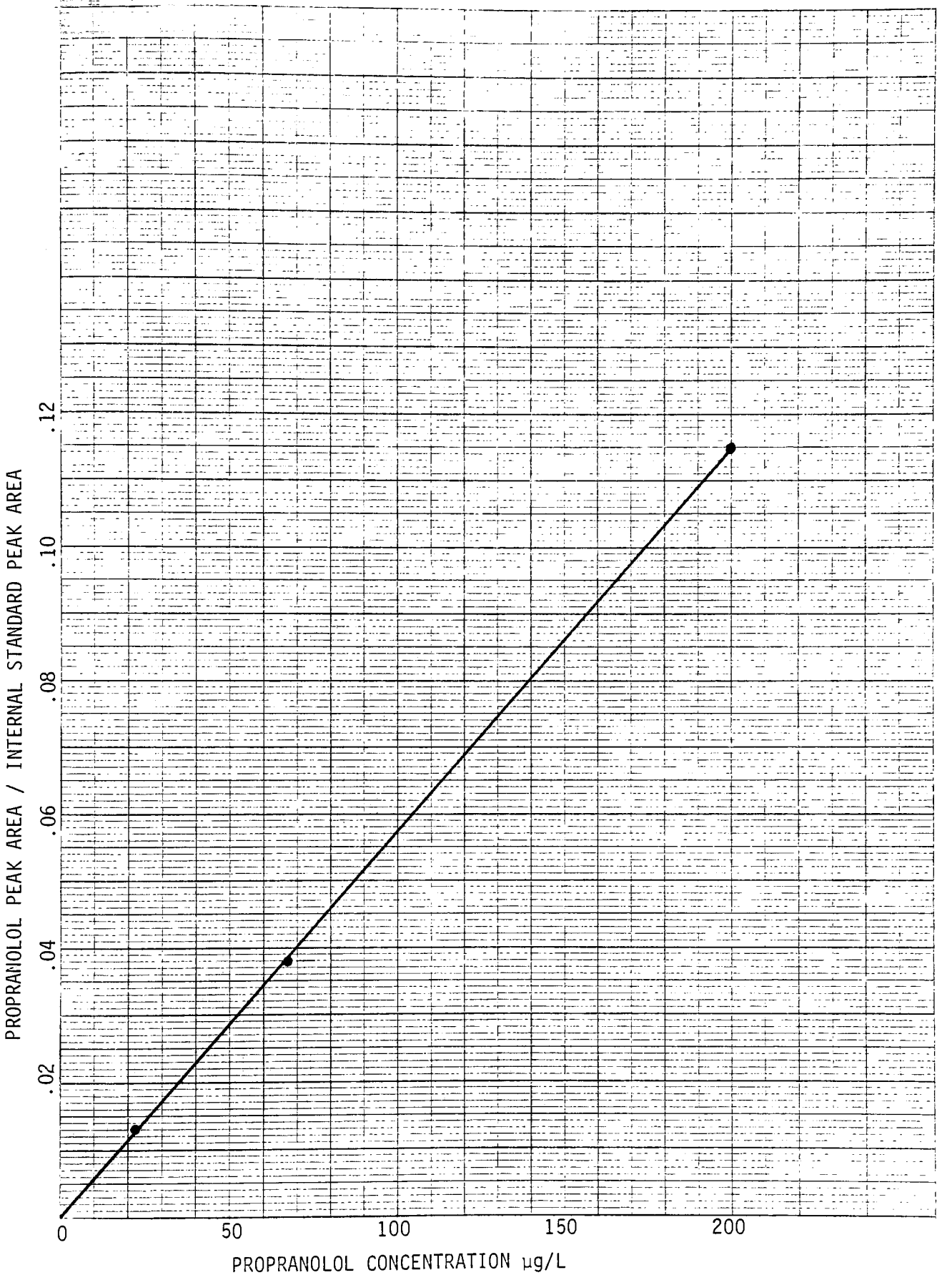

Fig. 8. Propranolol calibration curve from spiked serum extracts. Comparison of peak area drug/peak area internal standard with drug concentration in µg/L. Each point represents an average of three determinations. Standards are: 200 µg/L, 67 µg/L, and 22 µg/L.

TABLE 2. LINEAR REGRESSION ANALYSIS OF 3 POINT CALIBRATION CURVES FROM SPIKED SERA[a]

	Slope	Y-intercept	Correlation Coefficient[f]
Procainamide[a]	0.2195	-0.0027	0.9990
N-acetylprocainamide[b]	0.1344	-0.0055	0.9991
Mono-N-dealkyldisopyramide[c]	0.1531	0.0024	0.9994
Disopyramide[c]	0.1470	-0.0012	0.9994
Quinidine[b]	0.3426	0.0513	0.9986
Propranolol[d]	0.5435	0.0008	0.9938
Dihydroquinidine[e]	0.3364	-0.0057	0.9998

[a] Ordinate: Peak area drug or metabolite/Peak area internal standard.
Abscissa: Concentration of drug of metabolite standards as indicated below.

[b] Standards: 1.3, 4.0, and 12 mg/L.

[c] Standards: 0.9, 2.7, and 8.0 mg/L.

[d] Standards: 22, 67, and 200 µg/L.

[e] Standards: 0.13, 0.4, and 1.2 mg/L.

[f] raw data used in calculation.

TABLE 3. ABSOLUTE EXTRACTION EFFICIENCIES FROM SPIKED SERA AT VARIOUS DRUG CONCENTRATIONS

CONCENTRATION: (mg/L)	16	8	4	2	1
Procainamide	98%	98%	96%	99%	85%
N-acetylprocainamide	74	74	73	72	63
Mono-N-dealkyldisopyramide	99	102	102	108	98
Disopyramide	103	104	103	114	104
Quinidine	100	103	102	112	103
CONCENTRATION: (mg/L)	1.4	0.70	0.35	0.18	0.09
Dihydroquinidine	98%	96%	97	107%	97%
CONCENTRATION: (µg/L)	250	125	63	31	
Propranolol	73%	70%	76%	72%	

run variation. Spiked serum for the run-to-run variation study was stored frozen in 3 mL aliquots. The spiked serum was analyzed fifteen times for within-run variation. The run-to-run assays were performed in duplicate, for fifteen days over a four week period.

The relative standard deviation for all drugs, in both precision studies, is less than 10% (Tables 4,5, and 6). Data in Table 7 indicates the variation in retention times over the four week period of the run-to-run precision study. By using the shifts in the internal standard retention time to correct for shifts in the time of elution of the assayed drugs, the reproducability was within ±0.1 min over the four week period. This is particularly significant because the microprocessor can then be easily programmed to identify peaks of interest by the relative retention times of analyte to that of any selected standard (9). Chromatograms of spiked sera are presented in Fig. 9.

INTERFERENCES

Several types of interferences were investigated in the study. Frequently used prescription drugs, over-the-counter drugs, and cardiac associated drugs were tested as potential exogenous interfering agents. Lipemic, icteric, and hemolyzed sera were tested for possible endogenous interferences. Heparin and EDTA were investigated as suitable anticoagulants. Dihydroquinidine, a contaminant of quinidine, was identified by thin layer chromatography and quantified by liquid chromatography.

Drug Interference

The drugs listed in Table 8 were tested for interference with the assay drugs. The drugs were dissolved in mobile phase and dispensed singly into 1.5 mL injection vials, at their respective high therapeutic serum concentrations. The concentration in the vial was made to approximate the concentration that would result from a hypothetical serum sample that was carried through the extraction procedure. 75 µL of solution from each vial was injected into the chromatograph. The resulting retention times were compared to the internal standard and antiarrhythmic drug retention times. Previous experience with the efficiency of the column indicated that drugs eluting within 0.5 min of any component to be assayed might interfere with the accurate quantification of that component. Table 9 lists the drugs that were tested

TABLE 4. WITHIN-RUN PRECISION DRUG CONCENTRATION mg/L (PROPRANOLOL µg/L)

PA	NAPA	NDAD	DISOP.	QUIN.	DIHYQ.	PROPL.
7.3	7.3	3.6	3.3	6.1	0.59	134
7.0	7.1	3.4	3.1	5.8	0.54	124
7.1	6.4	3.7	3.6	6.6	0.64	144
6.5	6.1	3.3	3.1	5.8	0.58	121
7.1	7.3	3.5	3.2	5.9	0.57	131
6.6	5.5	3.6	3.5	6.6	0.64	140
7.2	7.4	3.6	3.3	6.0	0.59	134
6.7	6.1	3.5	3.3	6.2	0.61	132
7.0	6.5	3.5	3.2	5.9	0.59	129
6.8	6.6	3.4	3.1	5.9	0.54	124
6.9	6.8	3.4	3.2	5.9	0.57	125
7.1	6.8	3.5	3.2	6.1	0.58	131
7.0	6.8	3.5	3.2	5.9	0.58	128
7.2	7.4	3.5	3.2	6.0	0.55	127
7.0	6.5	3.5	3.2	6.0	0.55	129

TABLE 5. RUN-TO-RUN PRECISION (AVERAGE OF DUPLICATES)
DRUG CONCENTRATION mg/L (PROPRANOLOL µg/L)

DATE	PA	NAPA	NDAD	DISOP.	QUIN.	DIHYQ.	PROPL.
8/15	5.8	5.4	3.9	3.9	6.4	0.60	94
8/19	6.1	6.1	4.0	3.8	6.3	0.59	92
8/20	6.4	6.7	4.0	3.9	6.4	0.60	98
8/22	6.0	6.0	4.0	3.9	6.5	0.60	96
8/24	6.2	6.6	4.2	4.0	6.6	0.73	98
8/26	6.0	6.2	4.2	4.2	7.0	0.76	101
8/27	5.5	5.3	3.8	3.7	6.2	1.19	97
9/4	6.0	5.9	4.2	4.1	6.8	0.75	94
9/7	5.7	5.4	4.1	4.2	6.7	0.71	106
9/8	6.3	6.6	4.3	4.2	6.7	0.72	91
9/9	6.0	6.3	4.1	4.0	6.4	0.71	103
9/10	6.1	6.1	4.2	4.1	6.6	0.77	107
9/11	5.2	4.7	3.8	3.7	5.8	0.69	95
9/12	5.9	5.9	4.0	3.9	6.2	0.69	-[a]
9/19	5.9	6.2	4.1	4.1	6.4	0.71	94

[a] Peak area not integrated properly.

TABLE 6. REPRODUCIBILITY FOR ANTIARRHYTHMIC DRUG DETERMANATIONS AND REPRESENTATION PEAK AREA RATIOS

	Within-Run Serum Pool (n=15)		
	Mean (mg/L)	Standard Deviation (mg/L)	Relative Standard Deviation (%)
Procainamide	7.0	0.23	3.4
N-acetylprocainamide	6.7	0.56	8.3
Mono-N-dealkyldisopyramide	3.5	0.10	2.7
Disypyramide	3.3	0.14	4.2
Quinidine	6.1	0.23	3.9
Propranolol	130[a]	6.1[a]	4.7
Dihydroquinidine	0.58	0.031	5.3

[a] Concentration µg/L.

	Run-to-Run Serum Pool (n=15)[a]			
	Mean (mg/L)	Standard Deviation (mg/L)	Relative Standard Deviation (%)	Peak Area Ratio, Drug: Internal Standard
Procainamide	5.9	0.30	5.1	1.22
N-acetylprocainamide	6.0	0.55	9.3	0.75
Mono-N-dealkyldisopyramide	4.1	0.15	3.7	0.59
Disopyramide	4.0	0.17	4.3	0.57
Quinidine	6.5	0.29	4.5	2.18
Propranolol	98[b]	5.0[b]	5.1	0.054
Dihydroquinidine	0.69	0.064	9.3	0.20

[a] Between assay variation was determined by assaying the pool in duplicate on 15 days over a four week period.

[b] Concentration µg/L.

TABLE 7. RETENTION TIMES RUN-TO-RUN PRECISION STUDY
VALUES OBTAINED OVER A 4 WEEK PERIOD

	R_t RANGE (MIN)	R_t RANGE (MIN) CORRECTED[a]
Procainamide	5.25 - 5.53	5.32 - 5.50
NAPA	7.11 - 7.59	7.32 - 7.49
NPP	10.47 - 10.85	---
NDAD	14.52 - 14.84	14.68 - 14.78
Disopyramide	17.03 - 17.37	17.18 - 17.31
Quinidine	18.04 - 18.54	18.28 - 18.44
Dihydroquinidine	19.61 - 20.10	19.85 - 20.01
Propranolol	21.30 - 21.82	21.53 - 21.71

[a] Corrected for shifts in internal standard retention within the run.

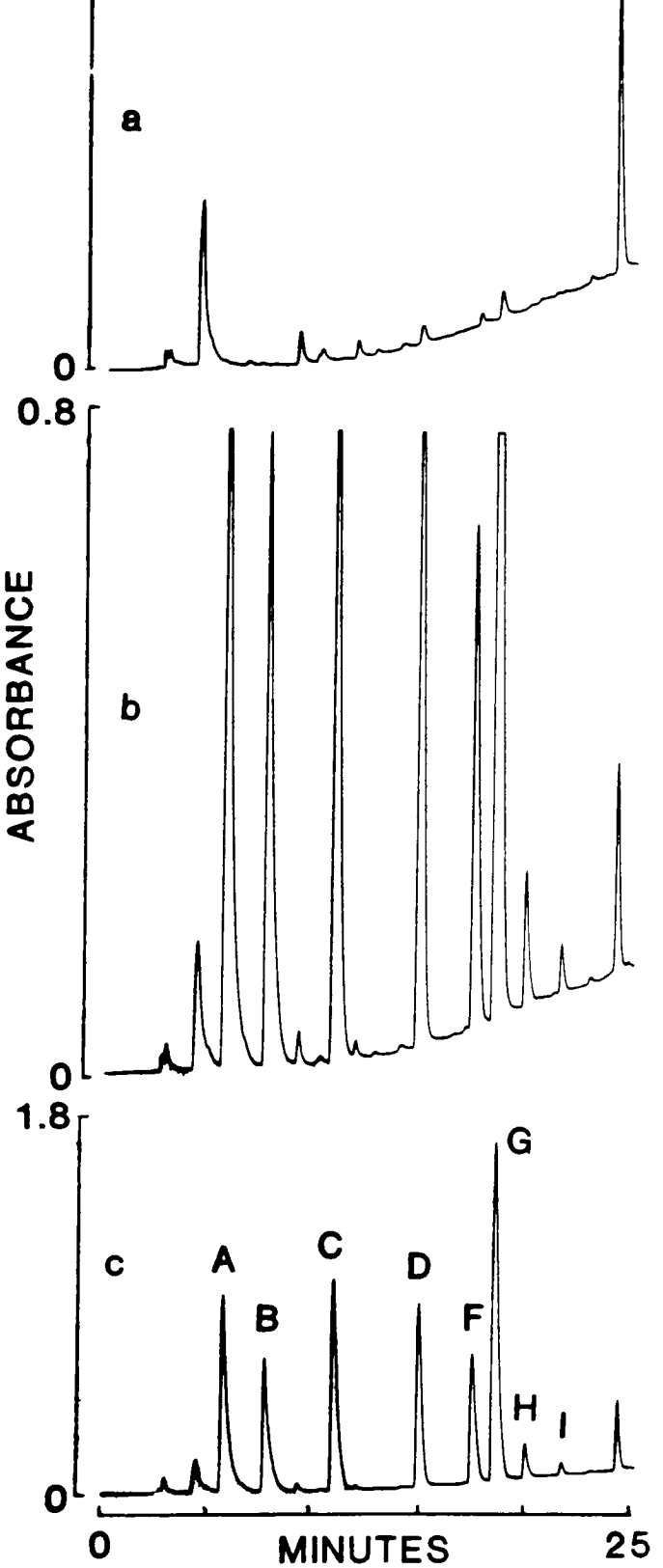

Fig. 9. Chromatograms for serum extracts of (a) blank serum, (b) spiked with drug at comparable detector sensitivity, and (c) serum spiked with drug at reduced sensitivity to illustrate peak shapes and relative heights. The concentrations are equal to those listed in Table 5 for run-to-run precision. The components are: A, procainamide; B, N-acetylprocainamide; C, N-propionylprocainamide; D, mono-N-dealkyldisopyramide; F, disopyramide; G, quinidine; H, dihydroquinidine; and I, propranolol.

TABLE 8. DRUGS TESTED FOR INTERFERENCE AT THE SERUM LEVELS INDICATED [a]

Anticonvulsants	
Phenytoin	20 mg/L
Phenobarbital	60 mg/L
Primadone	15 mg/L
Carbamazepine	10 mg/L
Valproic acid	100 mg/L
Analgesics	
Salicylate	400 mg/L
Meperidine	0.5 mg/L
Acetaminophen	40 mg/L
Pentazocine	0.3 mg/L
Propoxyphene	0.5 mg/L
Sedative / Hypnotics	
Flurazepam	1.0 mg/L
Glutethimide	4.0 mg/L
Methaqualone	5.0 mg/L
Meprobamate	20 mg/L
Psychotherapeutics	
Diazepam	2.0 mg/L
Chlordiazepoxide	10 mg/L
Oxazepam	1.0 mg/L
Amitriptylene	0.5 mg/L
Imipramine	0.5 mg/L
Doxepin	0.5 mg/L
Chlorpromazine	1.0 mg/L
Prochlorperazine	1.0 mg/L
Thioridizine	1.0 mg/L
Cardiac associated drugs	
Methyl Dopa	4.0 mg/L
Hydralazine	1.0 mg/L
Chlorthiazide	2.0 mg/L
Bretylium tosylate	1.0 mg/L
Isosorbide dinitrate	4.0 mg/L
Metaprolol	4.0 mg/L
Furosemide	10 mg/L*
Hydrochlorthiazide	4.0 mg/L*
Lidocaine	5.0 mg/L
Miscellaneous	
Theophylline	20 mg/L
Quinine	5.0 mg/L

* No data available on serum levels. Level calculated on recommended dose.

[a] Therapeutic concentrations found in References 1,10, and 11.

TABLE 9. INTERFERANCE STUDY: DRUG RETENTION TIME AND PEAK AREA

Drug	R_t [a]	Peak Area [b]
Amitriptylene	-	NR[c]
Imipramine	-	NR
Propoxyphene	-	NR
Chlorpromazine	-	NR
Thioridizine	-	NR
Prochloroperazine	3.2	335,400
Methyl Dopa	3.3	733,200
Hydralazine*	5.0	720,000
(Procainamide)	5.3	1,938,000
Theophylline*	6.0	10,660,000
Acetaminophen	6.3	7,036,000
Bretylium Tosylate*	6.9	149,100
(NAPA)	7.3	1,515,000
Chlorthiazide*	7.3	116,000
Hydrochlorthiazide	8.1	1,921,000
(NPP)	10.6	1,514,000
Salicylate	12.4	10,000,000
Bretylium Tosylate	12.7	219,600
Primadone	14.0	3,162,000
Lidocaine*	14.5	2,920,000
Meprobamate*	14.6	43,030
(NDAD)	14.7	309,300
Metaprolol	16.0	648,000
(Disoypramide)	17.2	956,000
Phenobarbital*	17.3	5,510,000
Furosemide*	18.0	1,583,000
Meperidine*	18.2	88,100
(Quinidine)	18.3	2,680,000
Quinine*	18.4	2,401,000
Pentazocine*	19.5	79,420
Valproic Acid*	19.7	174,000
(Dihydroquinidine)	19.9	196,900
Isosorbide Dinitrate*	19.9	833,600
P-Chlorodisopyramide	20.6	Internal std.
Carbamazepine*	20.9	4,440,000
Phenytoin*	21.0	2,799,000
Glutethimide*	21.3	1,767,000
Diazepam*	21.4	223,900
(Propranolol)	21.5	56,240
Flurazepam	22.4	538,900
Methaqualone	22.4	3,071,000
Oxazepam	22.7	626,300
Chlordiazepoxide	22.9	2,719,000
Doxepin	22.9	354,400

[a] R_t-retention time in min. [b] Area calculated by integrator at 212 nm.

[c] NR-no response (no detectable elution under assay conditions).

* Compound may interfere with an assay component because of its elution characteristics.

and their respective retention times. Those components identified with an asterik fulfill the criterion as possible interfering agents.

Separate aliquots of pooled drug free sera were individually spiked with the possible interfering drugs at the concentrations indicated in Table 8. Each was taken through the extraction procedure and injected into the chromatograph. Drug peak areas before and after extraction were compared to assess the extraction characteristics of each of the drugs.

To determine if consumption of quinine water (tonic water) would increase quinidine levels, a volunteer drank 830 mL (28 oz) of Schwepp's tonic water in a 15 min period. Blood was drawn 120 min later for determination of quinidine by our assay procedure.

A review of the drug extraction data from Table 10 shows the following; an apparent increase in procainamide concentration of 0.2 mg/L by hydralazine and an apparent increase in dihydroquinidine concentration of 0.06 mg/L by pentazocine, both of which are not clinically significant. Tonic water consumption would falsely increase quinidine by 0.1 mg/L, also insignificant. Quinine, a stereoisomer of quinidine, significantly interferes with the assay of quinidine. A 5.0 mg/L quinine level would increase quinidine by 5.0 mg/L. Lidocaine, a cardioactive drug with basic properties, will interfere with the quantification of NDAD.

The interference of glutethimide and carbamazepine, neutral extracting drugs, with propranolol, was investigated further. Three separate pooled drug free serum aliquots were spiked with either carbamazepine (10 mg/L), glutethimide (4.0 mg/L), or propranolol (100 µg/L). An aliquot of each of these sera was processed using the normal assay protocol. A second aliquot of each of the spiked sera was processed through to the aspiration of the organic eluting solvent. Instead of evaporating the HCl phase immediatedly after solvent aspiration, 8 mL of additional solvent was added to the tubes that contained 200 µL of HCl. The tubes were then vortexed and centrifuged again. The organic solvent was aspirated and then the HCl phase was evaporated to dryness. A third aliquot of spiked sera was taked through two additional solvent addition/aspiration steps. After the first 8 mL of new extraction solvent had been aspirated, another 8 mL was added. These tubes were then vortexed, spun, aspirated, and dried. During the aspiration step,

TABLE 10. EXTRACTION DATA ON COMPOUNDS WHICH MAY INTERFERE BECAUSE OF THEIR CLOSE ELUTION TO AN ASSAY DRUG

Drug	Serum conc. (mg/L)	Peak Area[b] Pre-extract	Peak Area[b] Post-extract	% Recovery
Chlorthiazide	2.0	119,200	1,100	0.9
Meperidine	0.5	521,900	NR[a]	0
Phenytoin	20	3,040,000	NR	0
Furosemide	10	3,610,000	NR	0
Meprobamate	20	40,200	NR	0
Diazepam	2.0	251,500	NR	0
Valproic acid	100	160,000	NR	0
Bretylium	1.0	120,600	NR	0
Isosorbide dinitrate	1.0	178,000	NR	0
Theophylline	20	12,835,000	NR	0
Phenobarbital	60	6,830,000	NR	0
Hydralazine	1.0	660,000	193,950	29.4
Lidocaine	5.0	2,739,000	2,503,000	91.4
Quinine	5.0	3,195,000	3,286,990	102.9
Carbamazepine	10	4,706,000	555,700	11.8
Glutethimide	4.0	1,944,000	193,060	9.9
Pentazocine	0.3	72,640	36,050	49.6
Tonic water	-	-	400,800	-

[a] NR-no response, no detectable elution under assay conditions.

[b] Area calculated by integrator at 212 nm.

approximately 7.5 mL of the 8 ml of organic solvent can be removed without disturbing the 200 µL HCl layer. In this way the initial extraction and back extraction steps remove 94% of neutral extracting compounds. A second solvent addition step increases the removal of these interfering drugs to 99.6%. A third addition would theoretically remove 99.98% of all neutral extracting compounds. The results of this experiment are tabulated in Table 11.

Both carbamazepine and glutethimide can be eliminated by the extra extraction steps. However, with each successive step there is a 30% loss of propranolol. After the third extraction, a 100 µg/L serum propranolol has a peak area of 25,000. This could present a detection problem with intermediate propranolol levels. There is no apparent loss of internal standard in these extraction steps. The 82% recovery of internal standard is therefore probably due to a loss of 18% in the column. A propranolol serum standard should therefore be taken through the multiple extraction steps along with the patient's specimen to compensate for extraction losses.

Endogenous Interference

Lipemic, icteric, and hemolyzed sera were tested for possible interferences with the assay drugs. Four pools were prepared of normal, lipemic, icteric,and hemolyzed sera. Aliquots of each were spiked with the assay drugs at concentrations to represent both high and low therapeutic serum levels:

	High	Low
Quinidine (mg/L)	5	2
Procainamide (mg/L)	5	2
NAPA (mg/L)	5	2
Disopyramide (mg/L)	4	2
NDAD (mg/L)	2	0.5
Propranolol (µg/L)	100	30
DIHYQ (mg/L)	0.6	0.2

Each specimen was then extracted in duplicate. Equivalent serum blanks were also run as controls.

All sera, regardless of whether or not a potential endogenous interference was present, had elution peaks at 4.57, 9.02, and 19.27 min. These substances, do not coelute with any of the assay drugs. Lipemic sera also had an endogenous peak at 12.34 min, area 175,000, that did not interfere with the assay. Another peak, that eluted 0.17 min after

TABLE 11. DATA ON THE ADDITIONAL EXTRACTION STEPS WHICH ARE REQUIRED TO REMOVE THE INTERFERING DRUGS CARBAMAZEPINE AND GLUTETHIMIDE FROM THE PROPRANOLOL ASSAY.

	Apparent Propranolol Level (µg/L)		
Extractions	Carbamazepine[a]	Glutethimide[a]	Propranolol[a]
Pre-extraction[b]	5129	2693	135
Extraction Protocol	733	178	99
One Added Extraction	39	0	73
Two Added Extractions	0	0	45

[a] Individual serum aliquots were spiked at the following concentrations; carbamazepine (10 mg/L), glutethimide (4 mg/L), and propranolol (100 µg/L).

[b] Drug standards were prepared in mobile phase at the indicated concentrations and chromatographed without extraction.

NDAD, produced the following areas in extractions of drug free sera: hemolyzed (22,100), lipemic (222,400), icteric (52,700). The extract of normal serum did not have this peak. A NDAD serum level of 0.5 mg/L produces a peak area of 170,000. Lipemic and icteric sera would significantly increase NDAD concentrations (Table 12) and should therefore not be used for analysis of this metabolite of disopyramide. Results of spiked sera (Table 12) show an elevation of NDAD levels in both lipemic and icteric specimens. Other results, such as the apparent decrease in propranolol in the low therapeutic spike of hemolyzed serum, could not be explained on the basis of the drug free serum data. Icteric sera also show an apparent procainamide increase. No explanation is offered for these data. Because of the problems related to the use of lipemic, icteric, and hemolyzed sera, they should not be accepted as appropriate samples for analysis of the antiarrhythmic drugs.

EDTA plasma and heparinized plasma were tested for suitability in assaying the antiarrhythmic drugs. Seventy ml of whole blood, one heparinized tube and one tube containing EDTA were drawn from a volunteer. Approximately sixty ml of the whole blood sample was quickly added to five ml of normal saline that had been spiked with drugs. The blood was mixed and transferred to tubes that contained either EDTA, heparin, or no anticoagulant. All serum and plasma samples were removed from the cells within one hour. The concentrations of spiked drugs were approximately as follows: quinidine 5 mg/L, procainamide 5 mg/L, NAPA 5 mg/L, disopyramide 4 mg/L, NDAD 1 mg/L, dihydroquinidine 0.4 mg/L, and propranolol 100 µg/L. Each of the three specimen types was extracted in duplicate. Blanks of each specimen type were also analyzed to determine if any anticoagulant related interferences are present. No interfering peaks were seen upon extraction of the blank serum or either of the plasma. It appears from the data in Table 13 that EDTA plasma, heparinized plasma, or serum may be used for all drugs except NAPA. EDTA plasma increases NAPA values and should not be used for assay of the procainamide metabolite. The reason for this increase could not be explained on the basis of the results of the drug free extracts. No explantion is offered for this increase.

Dihydroquinidine was identified as a contaminant of the quinidine sulphate preparation in two ways. First, the retention times of a known

TABLE 12. ENDOGENOUS INTERFERENCES[a,b]

LOW CONCENTRATION

SERUM TYPE	PA	NAPA	NDAD	DISOP.	QUIN.	DIHYQ.	PROPL.
Normal	1.87	1.74	0.59	2.55	2.84	0.26	33.9
Hemolyzed	2.15	2.49	0.64	2.33	2.49	0.22	22.6
Icteric	2.29	2.06	0.74	2.62	2.92	0.23	30.6
Lipemic	2.00	1.71	1.38	2.60	3.15	0.25	29.5

HIGH CONCENTRATION

SERUM TYPE	PA	NAPA	NDAD	DISOP.	QUIN.	DIHYQ.	PROPL.
Normal	4.91	4.55	2.47	4.89	6.82	0.65	130
Hemolyzed	4.87	5.43	2.39	4.44	6.09	0.56	105
Icteric	5.54	4.89	2.88	5.49	7.65	0.68	124
Lipemic	5.04	5.12	3.16	4.57	6.69	0.60	104

[a] Apparent drug concentration (mg/L), propranolol (µg/L).

[b] Average value of duplicate samples.

TABLE 13. SERUM VS PLASMA SUITABILITY[a,b]

	PA	NAPA	NDAD	DISOP	QUIN	DIHYQ	PROPL
EDTA	5.00	7.84	0.99	5.37	5.51	0.49	72.5
Heparin	4.80	7.04	0.98	5.59	5.44	0.48	70.2
Serum	4.79	7.01	0.98	5.33	5.50	0.48	74.5

[a] Apparent drug concentration (mg/L), propranolol (µg/L).

[b] Average value of duplicate analyses.

solution of dihydroquinidine and the contaminant peak matched exactly. Secondly, 20 µg each of quinidine (and the contaminant), dihydroquinidine, and quinine (a stereoisomer of quinidine) were spotted separately on two silica plates for thin layer chromatography. The R_f's of the contaminant and dihydroquinidine, with two different mobile phase compositions, agreed within experimental error (Table 14). All substances were detected by both short wavelength UV fluorescence and iodoplatinate spray.

CORRELATION

Procainamide, NAPA, quinidine, and disopyramide were assayed in patient's sera by our liquid chromatographic method and compared to commercially available enzymeimmunologic techniques (EMIT) (13).

Sera for determination of procainamide, NAPA, and quinidine were collected and stored frozen at -15° C until the time of assay when each specimen was thawed and vortexed for 5 s to insure homogeneity. Pooled drug free sera was spiked with disopyramide at various concentrations. Each sample was assayed once by our liquid chromatographic method and in duplicate by enzymeimmunoassay. Results by enzymeimmunoassay that exceeded the method linearity for quinidine and disopyramide (both 8.0 mg/L), or for procainamide and NAPA (both 16.0 mg/L), were diluted with drug free serum and reassayed. Dihydroquinidine was added to drug free serum at concentrations of 0.5 mg/L and 1.0 mg/L and assayed in triplicate by EMIT. Dihydroquinidine is stated in the Syva brochure to increase a quinidine value of 2.0 mg/L by 30% when present at a concentration of 0.6 mg/L (i.e. both quinidine and dihydroquinidine are equally effective antigens in the technique).

Quinidine Correlation

Assay of dihydroquinidine spiked sera by EMIT gave the following results:

Dihydroquinidine Added	Quinidine Found
1.0 mg/L	1.0 mg/L (average)
0.5 mg/L	0.5 mg/L (average)

Dihydroquinidine exhibits 100% cross-reactivity with the EMIT assay antibody for quinidine. Because of this cross-reactivity and the ability of the liquid chromatographic method to resolve the two drugs, the correlation was performed using quinidine (HPLC) vs quinidine

TABLE 14. THIN LAYER CHROMATOGRAPHY OF QUINIDINE (AND CONTAMINANT), DIHYDROQUINIDINE, AND QUININE

Mobile Phase[a]	R_f[b] Quinidine	(Contaminant)	Dihydroquinidine	Quinine
68:8:4	0.50	(0.44)	0.43	0.41
68:8:8	0 .57	(0.51)	0.52	0.49

[a] Volume of ethyl acetate:methanol:NH_4OH (conc.).

[b] Compared to mobile phase migration of 13.5 cm. Detection by short wavelength UV fluorescence after H_2SO_4:abs. ethanol (90:10) spray and also by iodoplatinate spray (Reference 12).

(EMIT), and quinidine plus dihydroquinidine (HPLC) vs quinidine (EMIT). The correlation data are found in Table 15 and Figure 10. The correlation is only slightly affected by the inclusion of dihydroquinidine in the calculations. Least square regression analysis of the two sets of data:

Quinidine + Dihydroquinidine (HPLC) vs Quinidine (EMIT)

r = .9508 HPLC = 1.11 (EMIT) - 0.13 n = 25

Quinidine (HPLC) vs Quinidine (EMIT)

r = .9554 HPLC = 1.08 (EMIT) - 0.14 n = 26

This is probably due to the low levels of dihydroquinidine in serum. The liquid chromatographic assay for quinidine correlates well with enzyme-immunoassay technique. The correlation coefficient, slope, and Y-intercept are all acceptable for clinical use. At present, there is insufficient data on the clinical significance of dihydroquinidine (14,15). We are reporting quinidine values and keeping records of both compounds for possible future use.

Procainamide / NAPA

The correlation between HPLC and EMIT for procainamide and NAPA, as well as the least square regression analysis of the data, are found in Figures 11 and 12, and Table 16. The ratio of NAPA to procainamide may reflect the metabolism of procainamide. In patients on chronic procainamide therapy, high ratios indicate fast acetylation while, low ratios indicate slow acetylation. Acetylation is genetically determined (1). Very high ratios also may indicate renal insufficiency due to the accumulation of NAPA. NAPA has a half life of 6 hours compared to 3 hours for procainamide (1).

Disopyramide / NDAD

Because of the infrequent use of disopyramide compared to the other antiarrhythmics, patient specimens were difficult to obtain. The method correlation was therefore performed on pooled drug free sera which had been spiked with disopyramide at several concentrations. The correlation between HPLC and EMIT for disopyramide, as well as the least square regression analysis of the data are found in Figure 13 and Table 17. There is no readily available comparative method for the analysis of NDAD. Quantification of NDAD may prove valuable because of its reported high cholergenic potency (16). Reported serum levels of

TABLE 15. COMPARISON OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ENZYMEIMMUNOASSAY FOR THE DETERMINATION OF QUINIDINE

Specimen	EMIT (mg/L)[a]	HPLC (mg/L)[b] Quin + Dihyq	Quin	Dihyq	Dihyq % Total[d]
2643	2.5	2.8	2.6	.16	5.8
6643	0.6	0.6	0.6	.01	2.0
8394	3.3	3.3	3.3	.05	1.5
3243	3.4	3.1	3.0	.07	2.3
2449	3.1	3.5	3.3	.20	5.7
8793	2.5	2.2	2.2	.03	1.3
6527	0.4	0.3	0.3	.02	6.3
3252	1.5	1.4	1.4	.04	2.8
2885	2.9	3.4	3.3	.14	4.1
5454	2.2	2.1	2.0	.09	4.3
4251	3.2	3.7	3.6	.10	2.7
105	1.7	0.7	0.7	.04	1.7
2976	4.0	4.5	4.4	.10	2.6
1607	5.0	5.5	5.3	.18	3.3
3042	2.8	3.1	2.9	.21	6.7
2251	2.4	2.5	2.3	.22	8.7
4622	2.1	2.0	1.9	.05	2.6
3753	2.3	2.0	2.0	c	-
2843	1.9	2.1	2.0	.08	3.8
4439	2.5	2.6	2.5	.06	2.3
3986	1.8	2.7	2.7	.04	1.5
2710	1.4	1.4	1.3	.12	8.5
9264	1.2	1.5	1.4	.08	5.4
4010	0.9	1.1	1.0	.07	6.5
5438	3.2	3.3	3.2	.08	2.4
4220	2.6	3.2	3.1	.09	2.8

[a] Average of duplicate assays. EMIT crossreacts 100% with dihydroquinidine (see text). EMIT result is therefore a sum of quinidine and dihydroquinidine.

[b] HPLC is capable of quantitating both quinidine and dihydroquinidine. Dihydroquinidine levels shown to illustrate its low concentration in patient serum.

[c] Peak area not integrated properly, therefore not included in the correlation

[d] Dihyq % Total =(Dihyq/(Dihyq + Quin)) x 100.

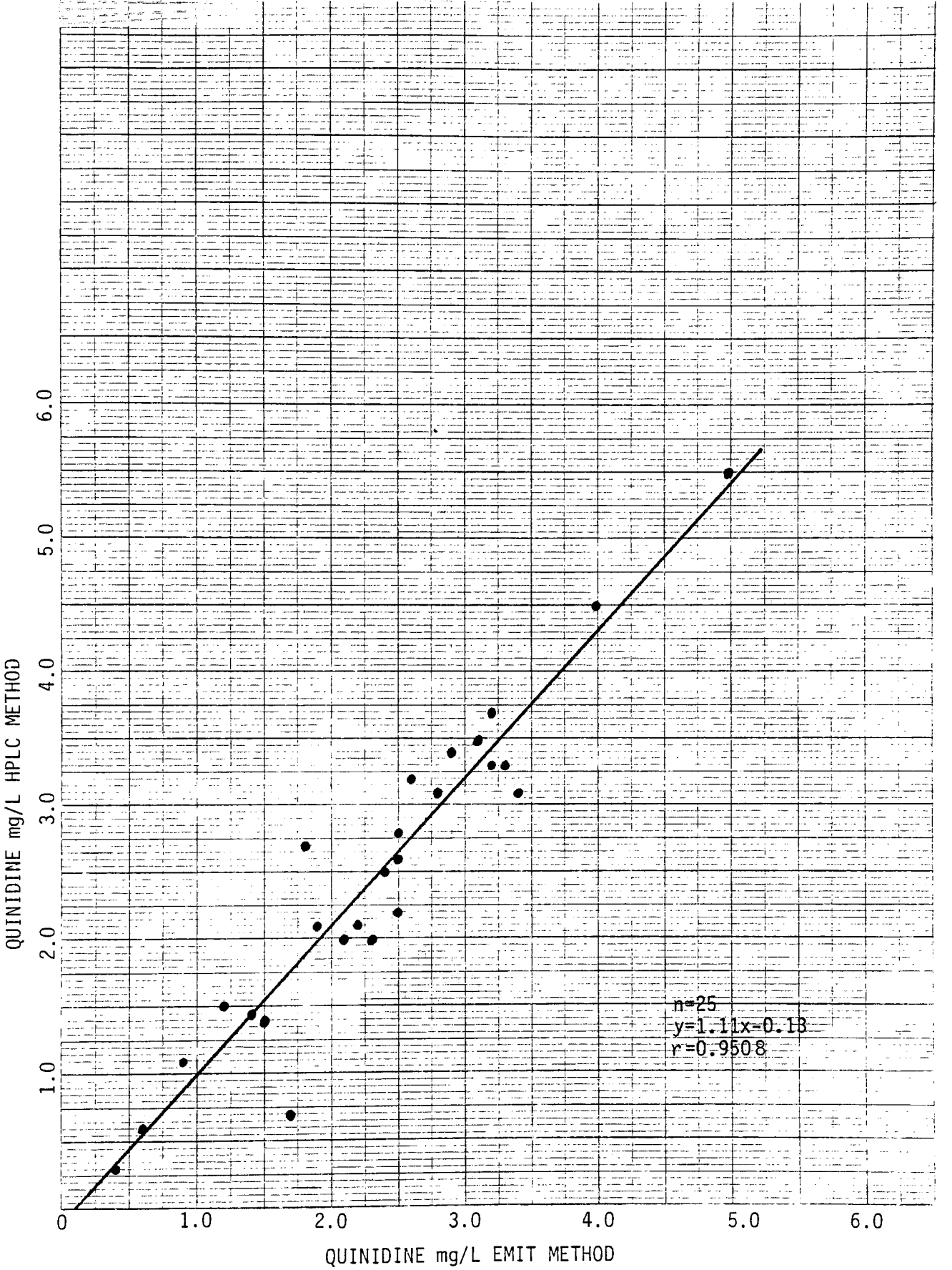

Fig. 10. Comparison of the high performance liquid chromatograph method for quinidine (Y-axis) with enzymeimmunoassay (X-axis).

TABLE 16. COMPARISON OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ENZYMEIMMUNOASSAY FOR DETERMENATION OF PROCAINAMIDE AND N-ACETYLPROCAINAMIDE

	Procainamide (mg/L)		NAPA (mg/L)			
Sample #	EMIT[a]	HPLC	EMIT[a]	HPLC	NAPA/PA[b]	NAPA & PA Total[c]
A-1	14.5	15.8	d	d	-	d
A-2	13.6	14.5	16.5	15.3	1.1	29.8
A-3	4.7	4.7	6.7	6.8	1.4	11.5
A-4	1.3	1.3	1.8	1.8	1.4	3.1
A-5	2.9	2.3	3.5	2.9	1.3	5.2
A-6	7.1	6.2	d	d	-	d
A-7	1.0	0.9	3.9	3.8	4.2	4.7
A-8	2.5	2.5	2.5	2.3	0.9	4.8
A-9	2.5	2.3	5.4	3.9	1.7	6.2
B-1	6.0	4.4	d	d	-	d
B-2	2.8	2.5	5.0	3.8	1.5	6.3
B-3	4.2	3.8	3.8	2.9	0.8	6.7
B-4	1.3	1.0	1.8	1.2	1.2	2.2
B-5	5.8	5.7	6.7	5.8	1.0	11.5
B-6	3.8	4.0	2.6	2.4	0.6	6.4
B-7	5.9	5.8	6.4	6.1	1.1	11.9
B-8	6.3	5.6	5.0	3.9	0.7	9.5
B-9	1.6	1.2	1.3	0.8	0.7	2.0
C-1	0.5	0.5	1.0	0.8	1.6	1.3
C-2	2.8	2.5	2.3	1.5	0.6	4.0
C-3	5.3	5.0	5.5	3.5	0.7	8.5
C-4	8.3	8.3	d	d	-	d
C-5	10.3	12.5	10.4	11.0	0.9	23.5
C-6	3.4	3.6	2.5	1.7	0.5	5.3
C-7	14.0	14.7	6.4	5.4	0.4	20.1
C-8	4.3	3.8	5.0	4.1	1.1	7.9
C-9	6.2	6.6	8.9	8.1	1.2	14.7

[a] Average of duplicate assays.
[b] Useful for determining the rate of acetylation (see Reference 1).
[c] For optimum patient management procainamide as well as the sum of procainamide and NAPA, should be reported (see Reference 1).
[d] Values exceeded the tested method linearity, not included in the correlation

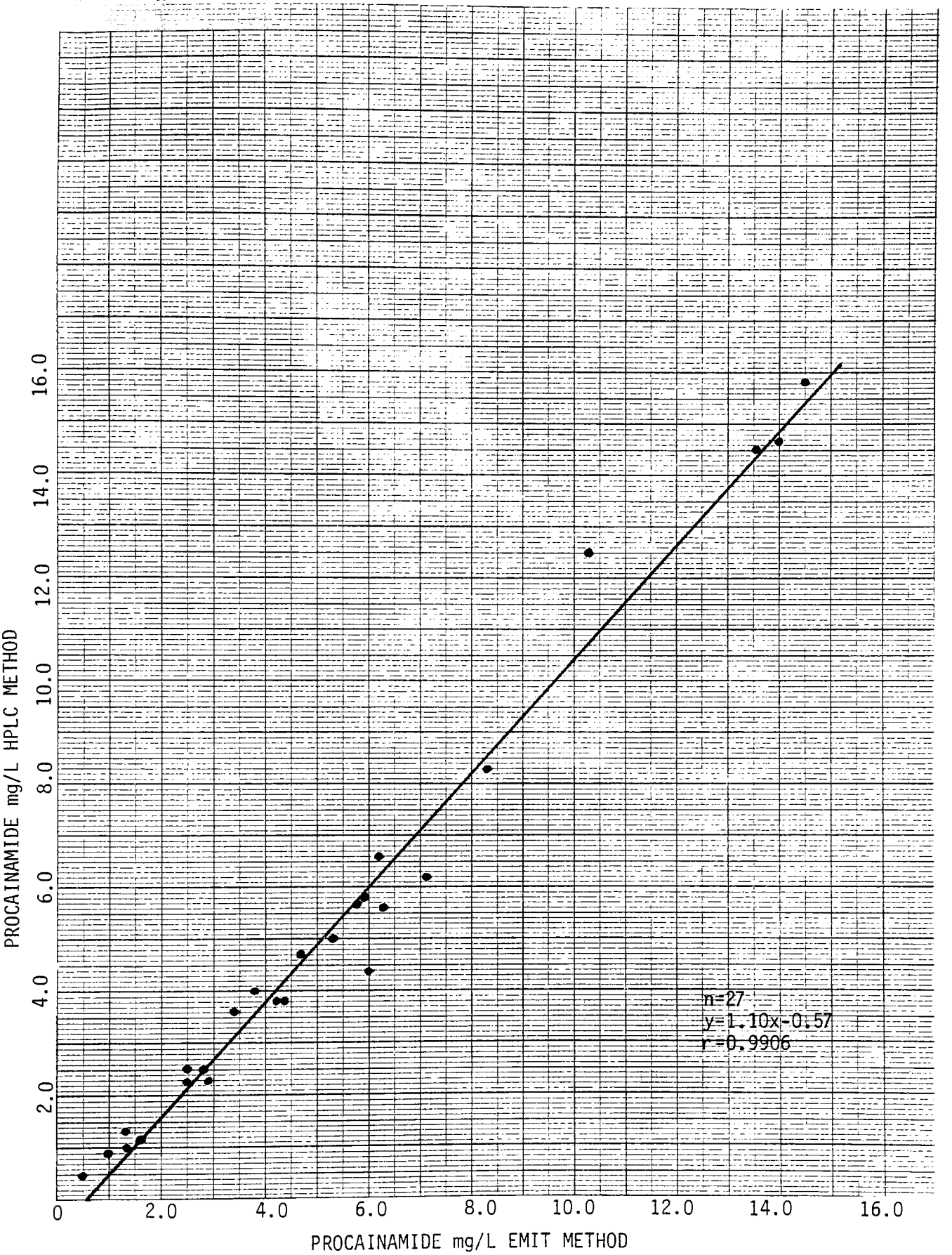

Fig. 11. Comparison of the high perfromance liquid chromatographic method for procainamide (Y-axis) with enzymeimmunoassay (X-axis).

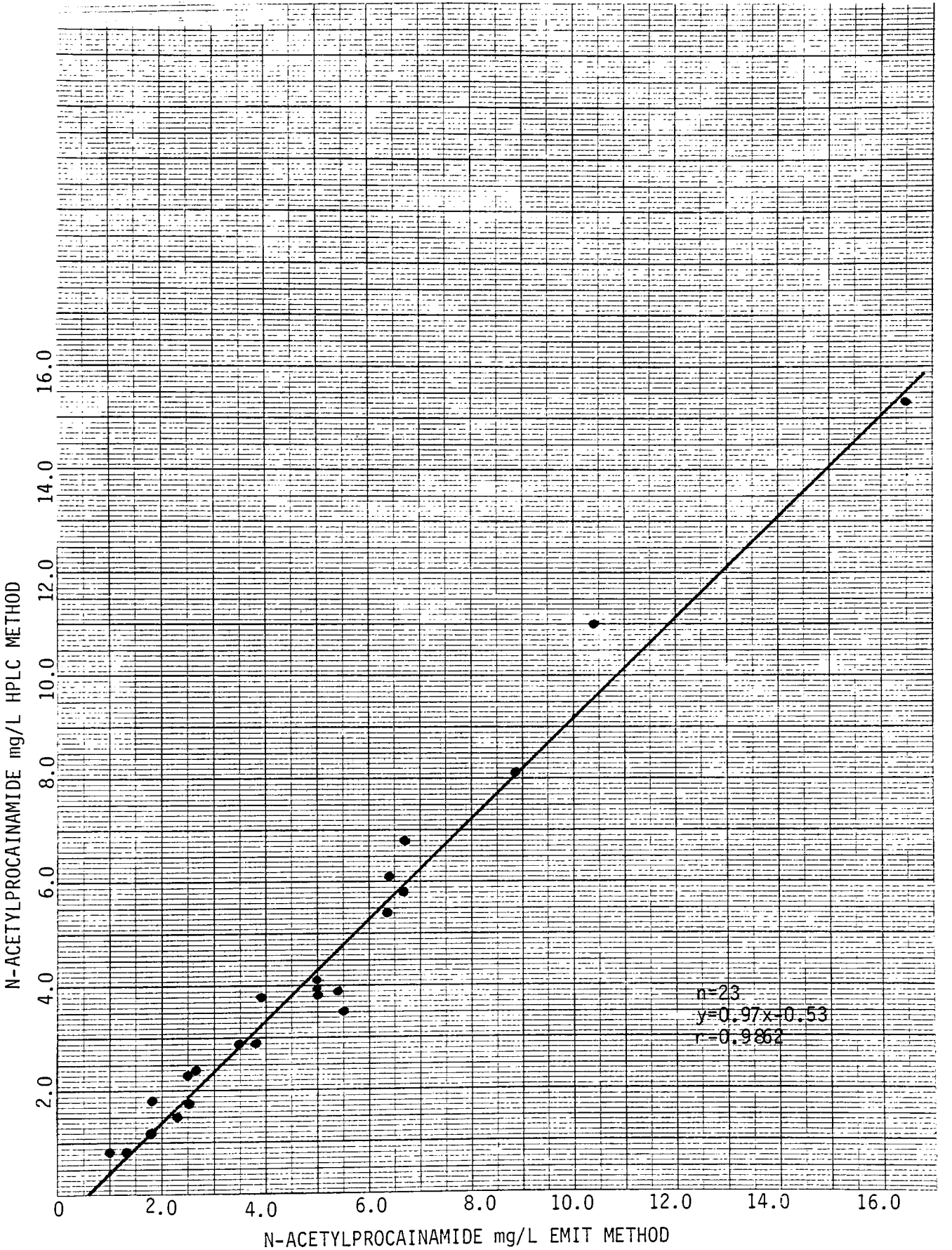

Fig. 12. Comparison of the high perfromance liquid chromatographic method for N-acetylprocainamide (Y-axis) with enzymeimmunoassay (X-axis).

TABLE 17. COMPARISON OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ENZYMEIMMUNOASSAY FOR THE DETERMINATION OF DISOPYRAMIDE

Specimen[a]	EMIT[b]	HPLC
A-1	0.5	0.6
A-2	1.0	1.0
A-3	1.6	1.5
A-4	2.2	2.3
A-5	2.1	2.3
A-6	2.7	2.9
A-7	2.6	2.2
A-8	3.3	3.2
A-9	3.8	3.3
B-1	4.0	3.6
B-2	4.4	4.2
B-3	4.5	4.5
B-4	5.9	4.7
B-5	4.8	4.5
B-6	5.8	5.3
B-7	5.8	6.5
B-8	6.8	7.5
B-9	6.7	6.1
C-1	7.7	7.9
C-2	8.6[c]	10.6
C-3	9.7[c]	9.8
C-4	10.4[c]	11.5
C-5	13.0[c]	13.1

[a] Drug free serum was spiked with disopyramide at various concentrations.

[b] Average of duplicate assays.

[c] Values originally exceeded method linearity.

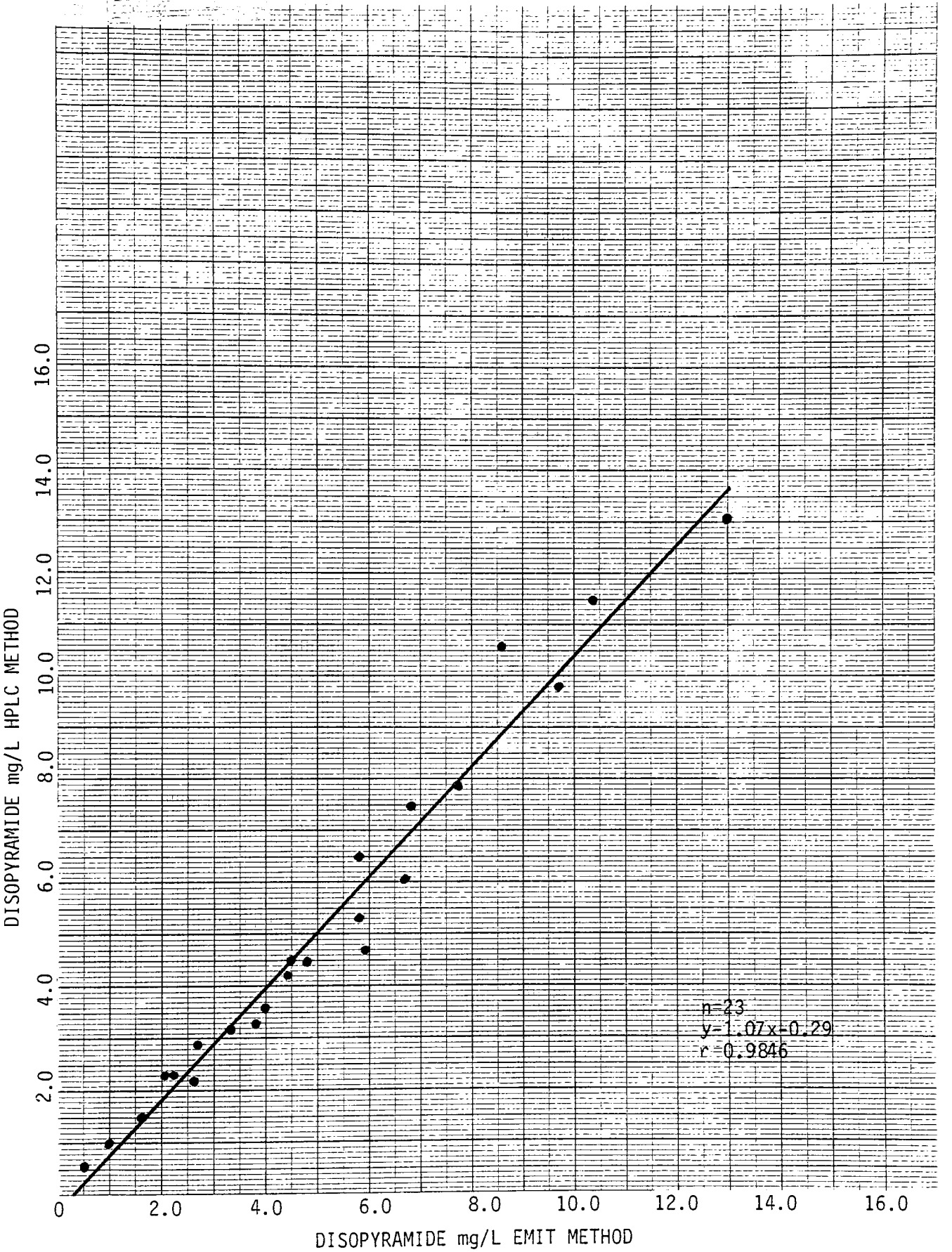

Fig. 13. Comparison of the high performance liquid chromatographic method for disopyramide (Y-axis) with enzymeimmunoassay (X-axis).

NDAD are within the linearity range of our assay, but the exact antiarrhythmic activity of this metabolite is uncertain (1,17). We are currently reporting disopyramide concentrations and measuring NDAD levels for possible future use.

Propranolol

At present, insufficient numbers of patient samples prevent us from acquiring correlation data for propranolol. The liquid chromatographic method for analysis of propranolol will be compared to our fluorometric method (4).

TIME COST STUDY

The time required to process individual runs of 4 specimens and 20 specimens was determined (Table 18). The total cost involved in the analysis of one specimen, when three specimens and one control are assayed, was also determined. These data are presented in Table 19.

There is a substantial savings in technologist time with the liquid chromatographic procedure compared to the methods formerly used. Assay of four specimens for propranolol by fluorometry requires 2.0 hours of technologist time (4). To assay the same number of patient sera for procainamide by spectrophotometry, 1.5 hours of technologist time is required (5). The total cost, however, is higher with liquid chromatographic analysis than with the manual methods. This is due primarily to instrument overhead. Enzymeimmunoassay, which is a precise and specific technique, is also an expensive method for analysis of these drugs.

The total cost of performing an EMIT assay for either quinidine, procainamide, NAPA, or disopyramide is $5.60 for a run of 4 patients (based on methodology applied to the Abbott ABA-100) (13). Liquid chromatography is therefore less expensive than EMIT when more than one drug is requested for assay on a patient's serum.

TABLE 18. TIME REQUIRED TO PROCESS THE NUMBER OF SPECIMENS INDICATED THROUGH THE ENTIRE ASSAY PROCEDURE

Step	Process 4 Specimens			Process 20 Specimens		
	Tech[a] Time (min)	Step[b] Time (min)	Total[c] (min)	Tech[a] Time (min)	Step[b] Time (min)	Total[c] (min)
1. Set up. Assemble all materials, dispense buffer, serum, and solvent twice.	12	12	12	35	35	35
2. Add third solvent, allow columns to drain.	1	7	19	7	17	52
3. Vortex tubes (back-extraction).	2	2	21	12	12	64
4. Spin, aspirate solvent.	4	15	36	15	30	94
5. Evaporate, add mobile phase, and transfer to injection vial.	5	20	56	20	30	124
6. Run on chromatograph	12	120	176	12	600	724
7. Calculate and report results.	2	2	178	10	10	734
Totals:	38 min		3 hr	111 min		12.2 hr

[a] Represents time required for technologist to perform task.

[b] Represents time to complete task.

[c] Represents time from beginning of procedure.

TABLE 19. TOTAL COST INVOLVED IN THE ANALYSIS OF ONE SPECIMEN WHEN THREE SPECIMENS AND ONE CONTROL ARE ASSAYED.

Cost Summation[a]	Amount
Technologist time	$1.74 (@ $11.00/hr)[b]
Detector lamp use	$0.75[c]
Analytical column use	$0.55[d]
Elution solvent	$0.18
Extraction solvent	$0.08
Clin-Elute column	$0.80
Disposables	$0.40[e]
Instrument	$5.16[f]
Total	$9.66

[a] Includes the cost of assaying the control serum .

[b] Includes wages and benefits.

[c] (Lamp cost / Life time in hr) x hr / run.

[d] Column cost / number of injections.

[e] Paper, vial caps, etc.

[f] Based on 10 year depreciation and service contract cost.

METHOD DEVELOPMENT

The development of the HPLC antiarrhythmic drug assay consisted of initially investigating the chromatographic conditions that would affect drug elution from the analytical column. The instrument parameters were then studied in an attempt to optimize the assay for maximum sensitivity. The chosen system was then used for quantitation of the assay components. Several organic solvents and procedures were also tested for their ability to extract the antiarrhythmic drugs from spiked serum.

MOBILE PHASE DEVELOPMENT

Most of the experimental work involved in this study was directly related to the development and optimization of the mobile phase. The mobile phase conditions that were sought would separate and elute all components of interest in a reasonable length of time. Several parameters were investigated: Composition (percentages of methanol, acetonitrile, phosphate), pH, and phosphate concentration. Both isocratic and gradient systems were used to elute the drugs. Flow rate and column temperature were also investigated.

Working solutions of drug standards were prepared in a similar manner for all experiments. Methanol stock solutions of each drug were grouped into three vials. Drugs with similar retentions were put in separate groups to help prevent misidentification. Concentrations of 30 - 50 mg/L for each component were used to approach full scale recorder deflection at a setting of 0.768 absorbance units full scale (AUFS). The solvent composition of the vial always corresponded to the starting composition of the mobile phase being evaluated. In each of the experiments one of the following chromatographic conditions was varied while the others were held constant at the indicated values: flowrate 1.0 ml/ min., temperature ambient, phosphate buffer 25 mmol/L pH 3.5.

Mobile Phase Composition

The percentage composition of the organic component of the mobile phase was varied. The percent methanol:acetonitrile was changed from 100:0 to 0:100 in several steps. The ratios of methanol:acetonitrile that were used were 100:0, 80:20, 70:30, 65:35, 60:40, 55:45, 50:50, 40:60, 30:70, 20:80, and 0:100.

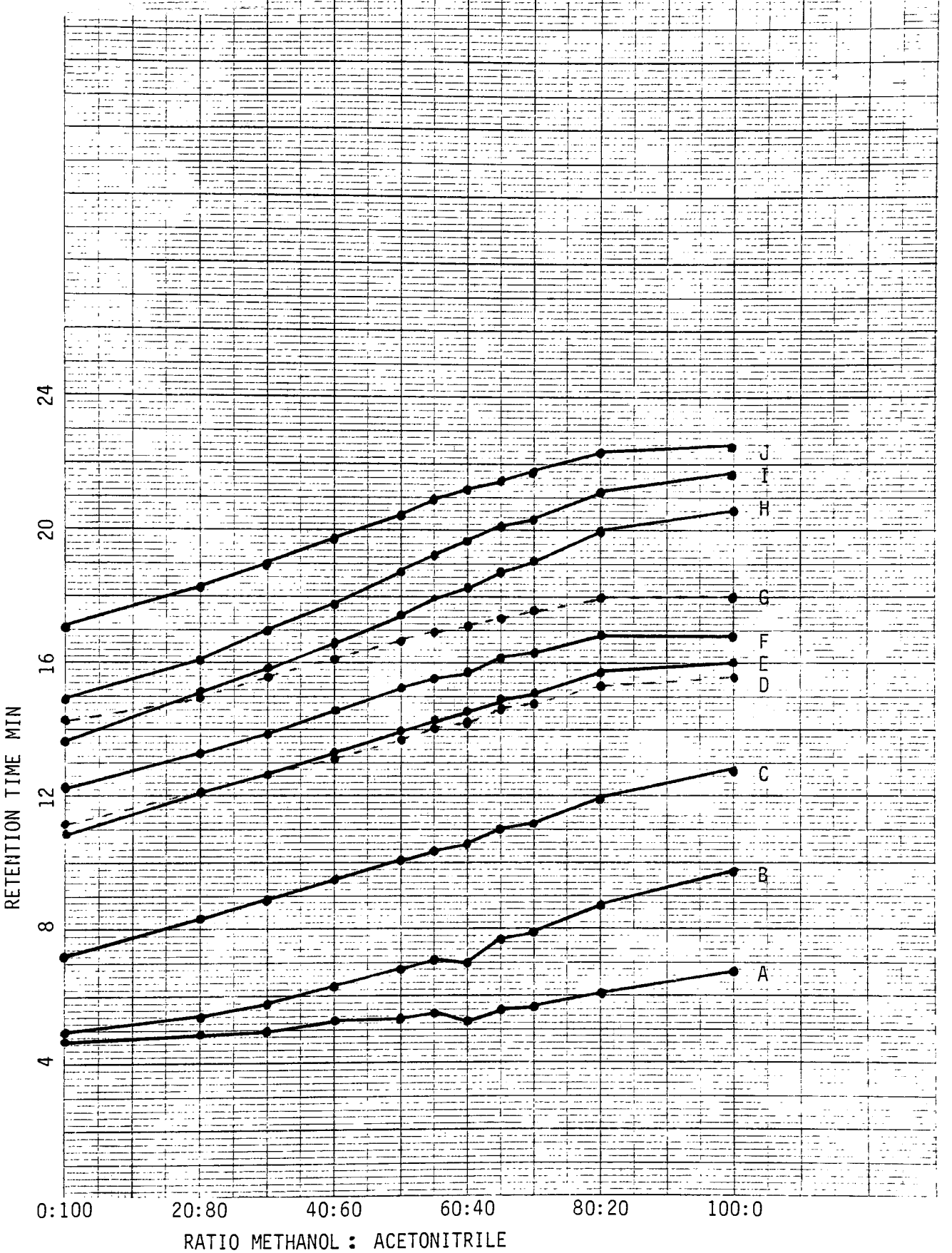

Fig. 14. Effect of the methanol:acetonitrile ratio of the mobile phase on drug retention. Drugs were eluted using gradient analysis in which the ratio of organic (methanol:acetonitrile)/phosphate changed from 20:80 at 2.0 min to 70:30 at 20 min. Components are: A, procainamide; B, NAPA; C, NPP; D, lidoc; E, NDAD; F, 4-OH propl; G, disop; H, quin; I, dihyq; and J, propl.

As shown in Figure 14 the retention times of all assay drugs increase directly with methanol concentration and, conversely, they decrease with increasing acetonitrile concentration. The change in retention time is variable from drug to drug, and results in changes in the elution order of some components. At methanol concentrations of 0 - 40% quinidine is not completely resolved from disopyramide. At 100% methanol, dihydroquinidine is not completely resolved from propranolol. For most assay drugs resolution improves as methanol concentration is increased from 40% - 80%.

The early peaks broaden and tail severely with high methanol concentrations (80 - 100%). The baseline shows an upward drift with time due to an increase in the absorbance that results from an increase in the gradient methanol concentration. At 100% methanol, the increase in base line absorbance represents a 20% full scale deflection at the end of the run (instrument attenuation set at 0.768 AUFS). Runs with acetonitrile concentrations greater than 60% have significantly smaller baseline increases. The baseline differences, although noticeable, are not analytically significant because they occur gradually, and thus do not affect the instrument's ability to properly integrate all of the peaks. The slight baseline increase occurring late in the run is due to increasing methanol in the mobile phase which is a function of the gradient design (see Experimental section, Chromatographic Conditions). Figures 15, 16, and 17 illustrate differences in the elution and baseline of six assay components at 100% methanol, at 100% acetonitrile, and at 60/40 methanol/acetonitrile, which was the ratio adopted for the assay. This ratio was chosen because all drugs were well resolved.

Phsophate Buffer pH

The effect of the buffer pH on the chromatographic elution of each drug was studied. Analyses were performed at pHs of 3.5, 4.5, 5.5, and 6.5.

In general, an increase in pH increases drug retention time (Figure 18). This is expected because all drugs in the study have basic properties:

Lidocaine pK = 7.85	Quinidine pK = 4.0,8.6
Disopyramide pK = 8.34	Procainamide pK = 9.2
Propranolol = 9.45	

(The pKs of NAPA, NDAD, and dihydroquinidine were not available.)

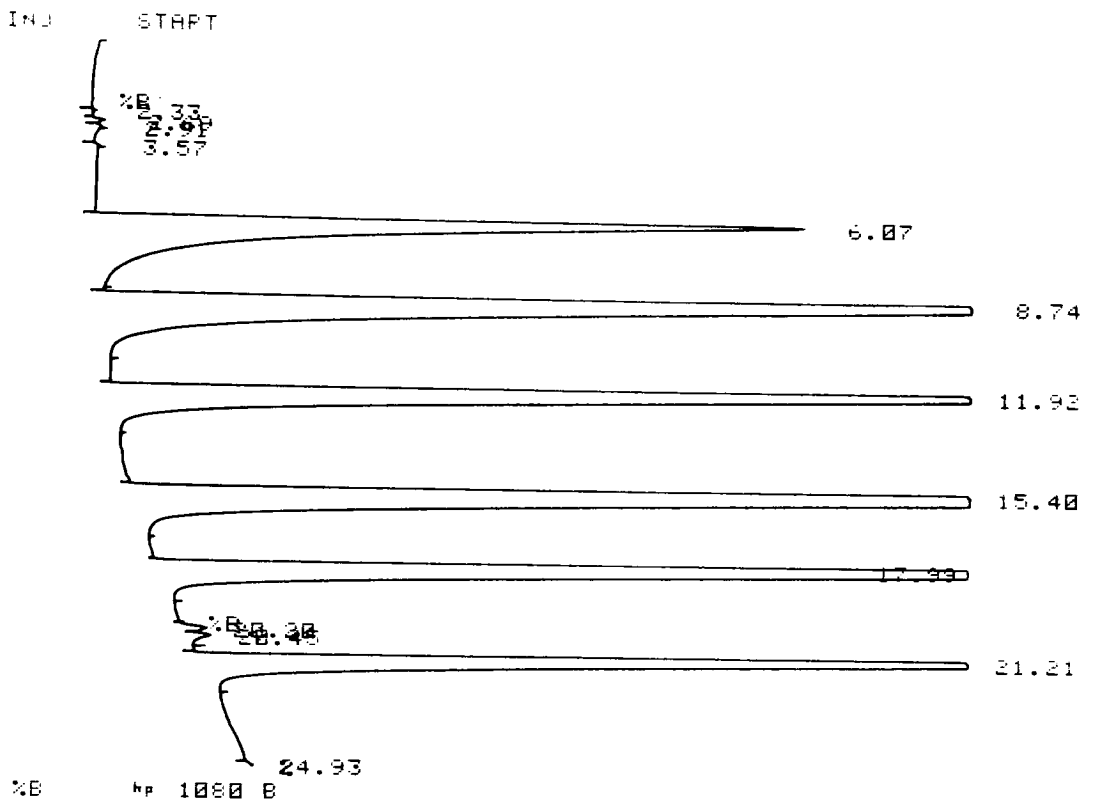

Fig. 15. Chromatogram of several assay components which were separated using a mobile phase organic ratio of 100% methanol:0% acetonitrile. The components with their respective retention times (min) are: procainamide (6.07); NAPA (8.74); NPP (11.92); lidoc (15.40); disop (17.99); and dihyq (21.21).

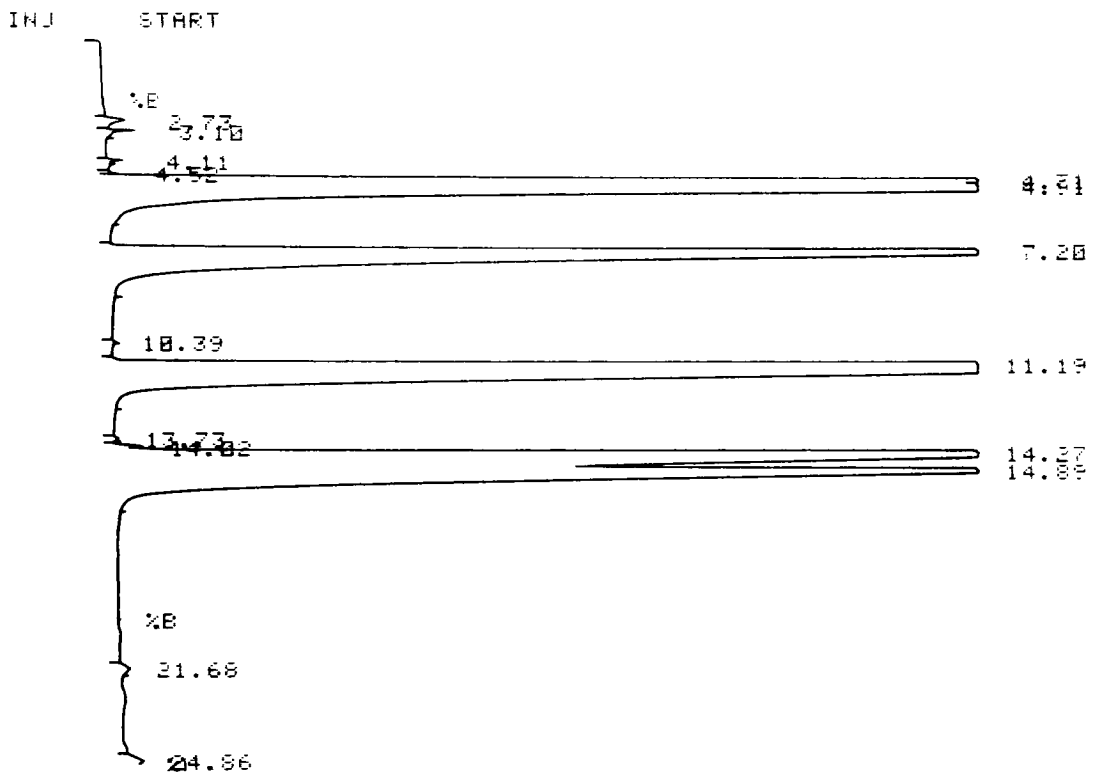

Fig. 16. Chromatogram of several assay components which were separated using a mobile phase organic ratio of 0% methanol:100% acetonitrile. The components with their respective retention times (min) are: procainamide (4.71); NAPA (4.91); NPP (7.20); lidoc (11.19); disop (14.27); and dihyq (14.89).

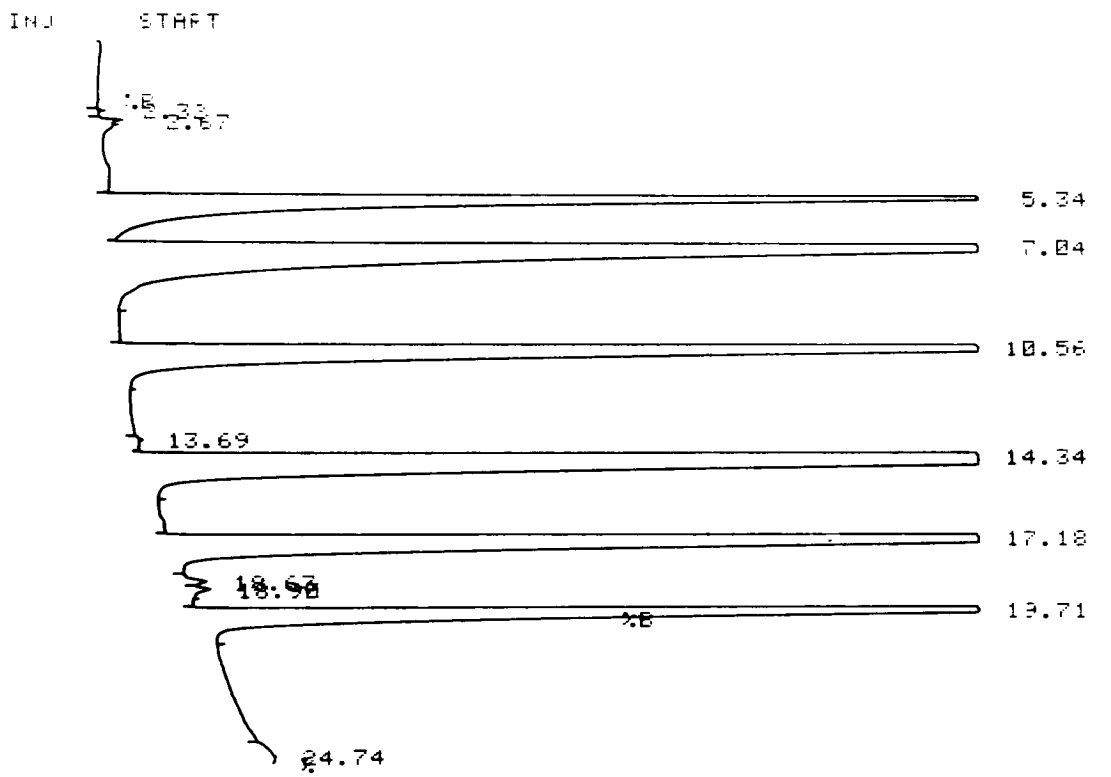

Fig. 17. Chromatogram of several assay components which were separated using a mobile phase organic ratio of 60% methanol:40% acetonitrile, (assay protocol conditions). The components with their respective retention times (min) are: procainamide (5.34); NAPA (7.40); NPP (10.56); lidoc (14.34); disop (17.18); and dihyq (19.71).

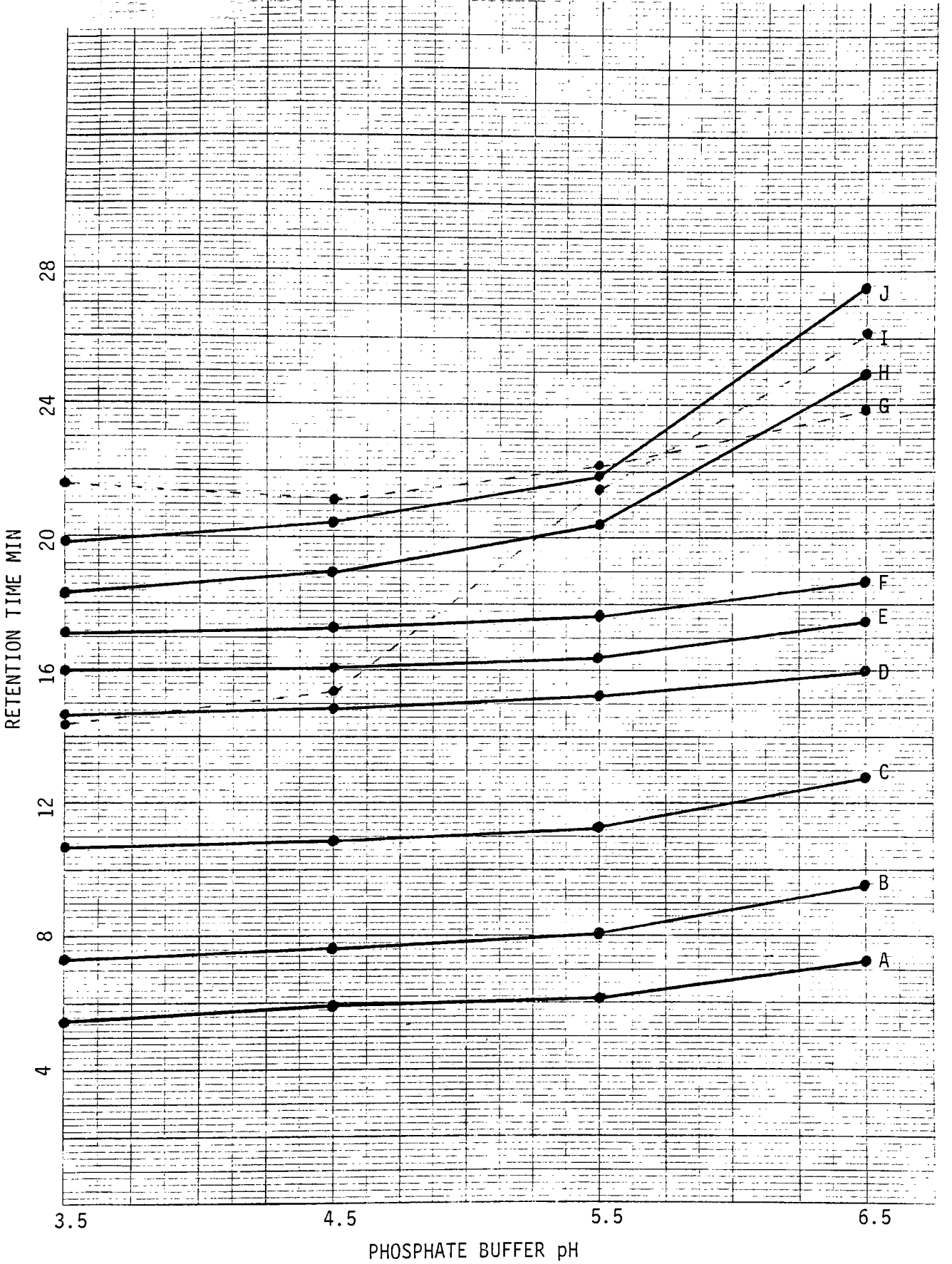

Fig. 18. Effect of phosphate buffer pH on drug retention. Components are: A, procainamide; B, NAPA; C, NPP; D, NDAD; E, 4-OH propl; F, disop; G, propl; H, quin; I, lidoc; and J, dihyq.

At low pH these drugs are in an ionized, hydrophilic form and they would be expected to elute quickly from the nonpolar, hydrophobic stationary phase. At high pH they are unionized and, hence, are retarded on the column. The large increase in the retention time of lidocaine is probably a result of the drug's relatively low pK and it's subsequent decrease in ionization at pHs 5.5 and 6.5. At the selected pH for the assay (3.5), all drugs, except NDAD and lidocaine, are separated by at least one min. As the pH increases, propranolol converges on dihydroquinidine. Further pH increases result in the propranolol converging on quinidine. Only at pH 6.5 are they again well separated. At pH 6.5, lidocaine is well resolved from NDAD. If lidocaine were to be included in the assay, this would be the appropriate pH.

Phosphate Concentration

The concentration of the phosphate buffer was varied over the concentration range of 12.5 mmol/L to 100 mmol/L. The optimum amount of phosphate would be the lowest concentration which would allow good resolution in a reasonable time, and still maintain an adequate buffering capacity. High phosphate concentrations may precipitate in the capillary lines of the liquid chromatograph (internal diameter of 0.0001 inch).

A slight increase in retention time for each of the drugs, is seen as the concentration is decreased from 100 to 25 mmol/L (Figure 19). A further decrease in concentration to 12.5 mmol/L results in a dramatic increase in drug retention. This effect could be due to a loss of buffering capacity at such low phosphate concentration. Elution from the nonpolar column is dependent upon the ability of the buffer to stabilize the charge on these basic drugs. If this ability is lost, the nonpolar drugs remain on the hydrophobic column. A phosphate concentration of 25 mmol/L was selected for the assay protocol. Drug elution times were acceptable and stable from run to run. Phosphate precipitation should not be a problem at this concentration.

Flowrate

The mobile phase flowrate was varied from 0.5 to 2.0 ml/min in several steps to determine the affect on the elution of all the assay components. The drugs were chromatographed at the following flowrates: 2.0, 1.75, 1.5, 1.25, 1.0, 0.75, and 0.5 ml/min.

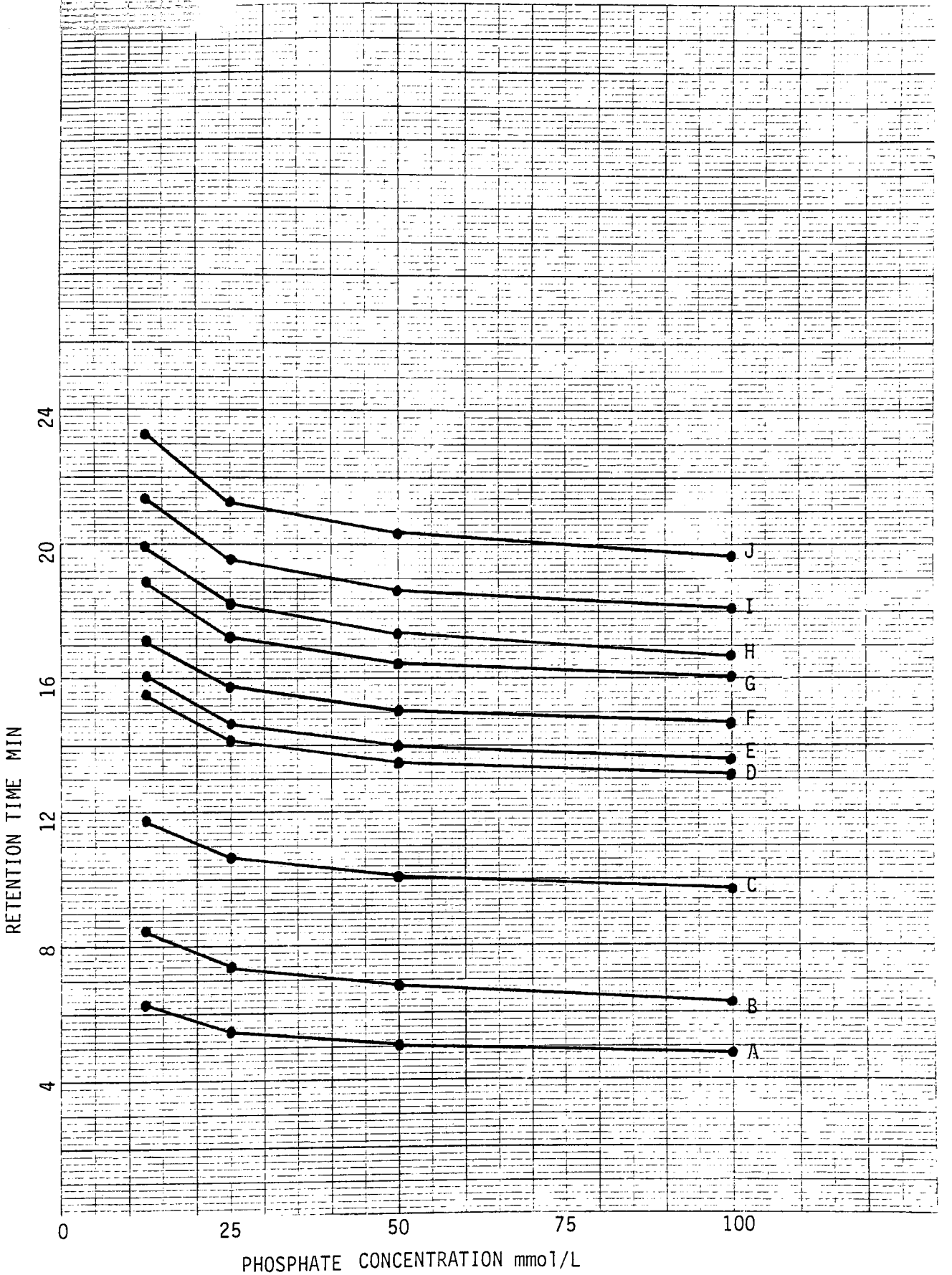

Fig. 19. Effect of phosphate buffer concentration on drug retention. Components are: A, procainamide; B, NAPA; C, NPP; D, lidoc; E, NDAD; F, 4-OH propl; G, disop; H, quin; I, dihyq; and J, propl.

As theory predicts (18), an increase in the flowrate shortened the elution time (Figure 20). Elution order was maintained throughout the range of the flowrate tested. Drug resolution, however, as defined in Figure 21, decreased directly with flowrate. A comparison of procainamide with disopyramide showed the following:

Flowrate (ml/min)	Resolution
2.0	4.90
1.0	3.53
0.5	2.82

An improvement in resolution at higher flowrates is illustrated by NDAD and lidocaine. These drugs coelute at a flowrate of 0.5 ml/min, but they separate at 2.0 ml/min. Peak areas were inversely proportional to flowrate. In all cases, an increase in the flowrate by a factor of two, decreased the peak area by approximately one-half. Noninterfering, endogenous peaks from serum components followed the same pattern. This behavior was thought to be related to the residence time of the absorbing material in the detector flowcell.

1.0 ml/min was selected as the flowrate for the assay protocol. Run length was acceptable and the column head pressure of 3000 psi was within the tolerance of the column. Although drug resolution is better at 2.0 ml/min than at 1.0 ml/min, a 5400 psi column head pressure is generated at the higher flowrate. At a given flowrate, head pressure varies ±15% depending on the condition of the filters in the flow system, the degree of deterioration of the pre-column, and the analytical column. At a flow of 2.0 ml/min, head pressure could exceed the maximum allowable system pressure of 6000 psi (9). This would result in system shutdown and possible column damage.

Column Temperature

Drugs were chromatographed at ambient, 35^{o}, 45^{o}, and 55^{o} C to determine the affect of temperature on the analysis. Flowrates of 1.0 and 0.5 ml/min were used at each temperature to determine if elution could be improved by the use of a high column temperature along with a low mobile phase flowrate.

At both flowrates, retention times were little affected by temperature between ambient and 35^{o} C. (Ambient temperature on the day of assay was 22^{o} C) (Figures 22 and 23). Because room temperature fluctuates between 20^{o} and 30^{o} C, the variation in elution times between 20^{o}

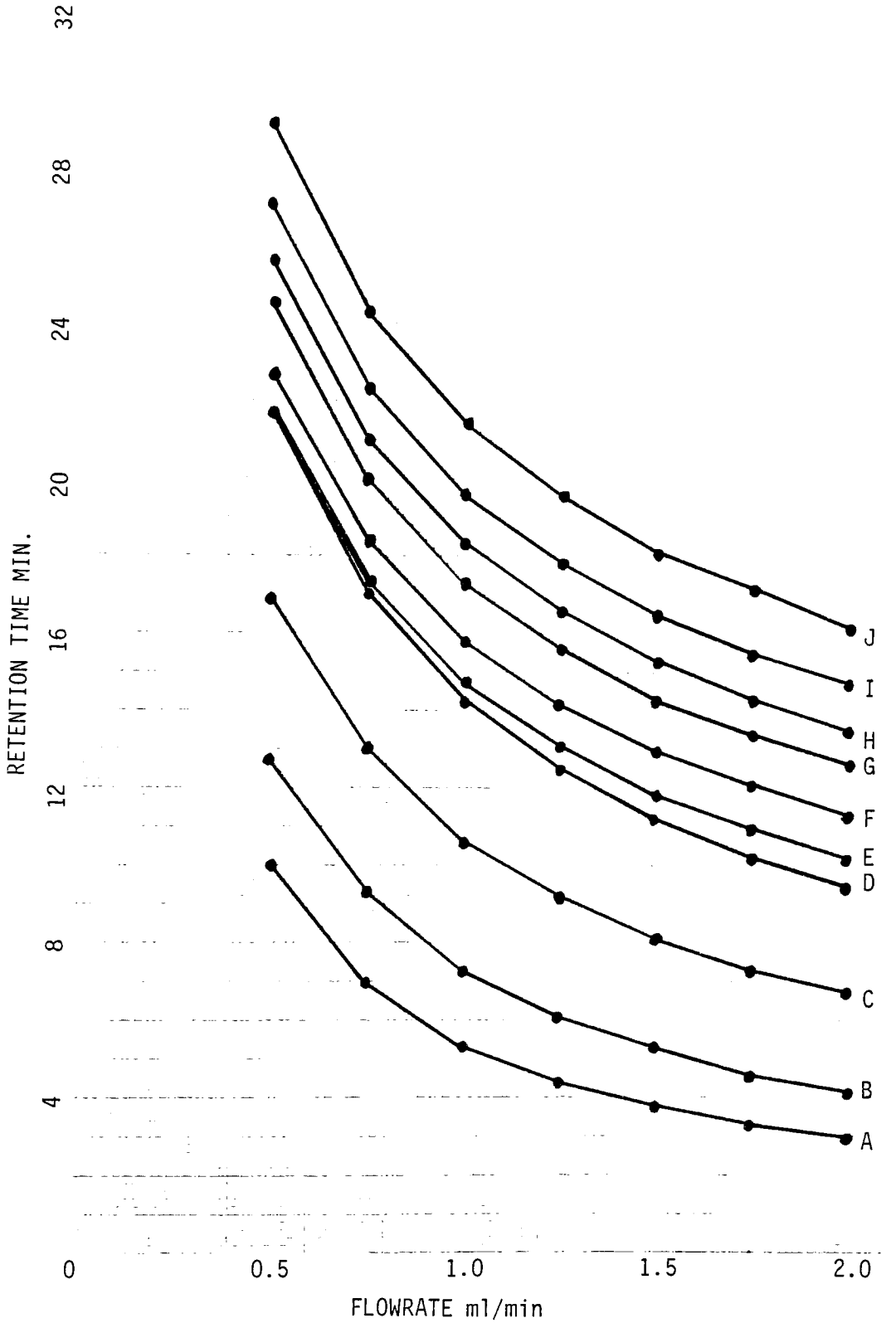

Fig. 20. Effect of mobile phase flowrate on drug elution. Components are: A, procainamide; B, NAPA; C, NPP; D, lidoc; E, NDAD; F, 4-OH propl; G, disop; H, quin; I, dihyq; and J, propl.

$$R = \frac{R_{t2} - R_{t1}}{\frac{W_1 + W_2}{2}}$$

Fig. 21. Equation for resolution of two components. R - resolution. $(R_{t2} - R_{t1})$ - difference in retention time between the two components, (retention time is measured at peak maximum). $((W_1 + W_2)/2)$ - average of peak widths measured at 10% above the baseline of each peak.

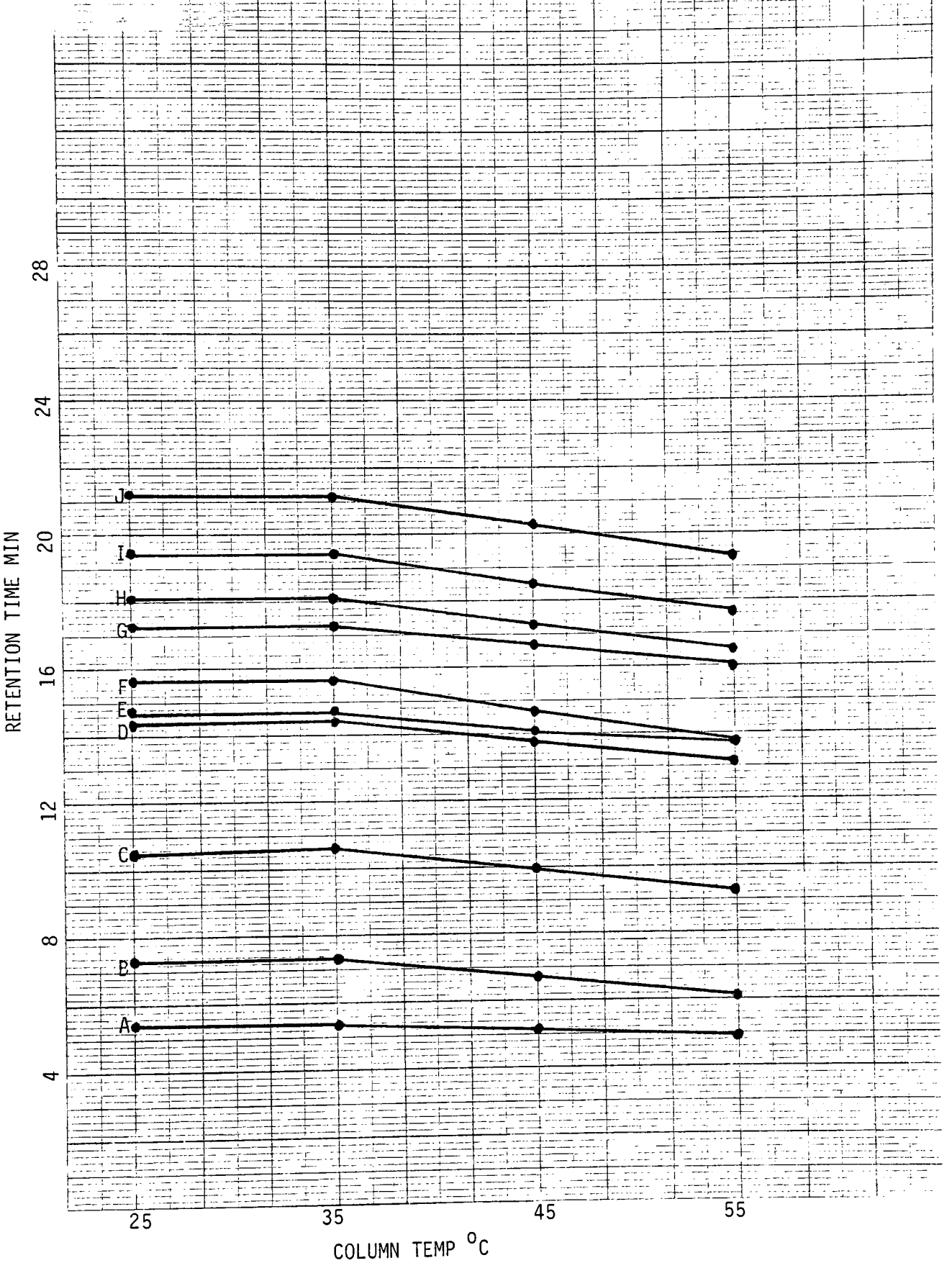

Fig. 22. Effect of column temperature on drug elution. Mobile phase flowrate 1.0 ml/min. Components are: A, procainamide; B, NAPA; C, NPP; D, lidoc; E, NDAD; F, 4-OH propl; G, disop; H, quin: I, dihyq; and J, propl.

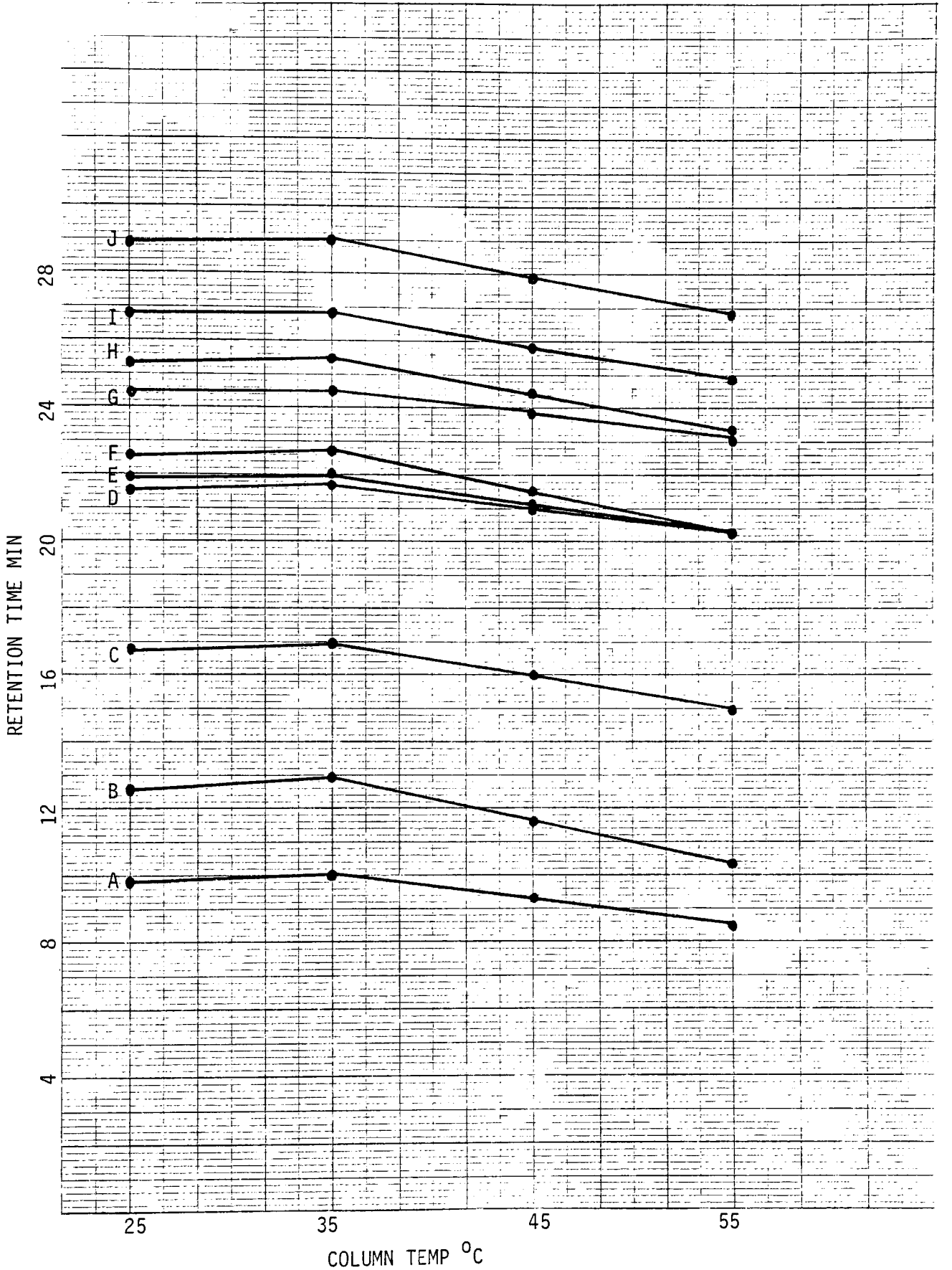

Fig. 23. Effect of column temperature on drug elution. Mobile phase flowrate 0.5 ml/min. Components are: A, procainamide; B, NAPA; C, NPP; D, lidoc; E, NDAD; F, 4-OH propl; G, disop; H, quin; I, dihyq; and J, propl.

and 35° C was considered especially important. The instrument is not capable of oven cooling, and when it is operated at ambient temperature the column oven is usually three degrees higher, due to heat generated by the instrument. However, the data indicate that changes in ambient temperature should not cause elution problems.

At temperatures over 35° C, drug retention time decreases and resolution worsens. At 55° C disopyramide and quinidine converge, as do NDAD, lidocaine, and 4-OH propranolol. Increasing the temperature beyond 35° C offers no advantage because of poor resolution. Ambient temperature was chosen for use in the assay.

Isocratic Elution

Isocratic elutions are generally reproducible and more readily useful for routine analysis. Therefore, all drugs were chromatographed in mobile phases of fixed composition to determine elution characteristics. All assay drugs were run at the following mobile phase ratios of organic:phosphate: 70:30, 60:40, 50:50, 40:60, and 30:70. The normal run time of 25 min was increased to 30 min to permit elution of as many drugs as possible from the column.

Isocratic elution at 30 - 40% organic modifier results in extreme tailing of the later eluting drugs and in poor separation of the earlier eluting components. Some drugs do not elute at all. Resolution of N-propionylprocainamide and propranolol, as determined by using the equation in Figure 21, is 1.59 at 40% organic. By comparison, the gradient elution program chosen for the assay protocol yields a R of 3.09 for the same two drugs. The retention times of these two drugs are approximately the same in both run. This illustrates the poor resolution and efficiency of the 40% isocratic run.

At 60 - 70% organic phase the peaks converge and have poor resolution (Figure 24). Interferences may coelute with the assay drugs, all of which elute within the first seven minutes.

An isocratic run with 50% organic modifier would be effective for the analysis of the antiarrhythmic drugs other than procainamide and NAPA. The remaining drugs separate well and have good peak symmetry. Isocratic analysis is, however, not appropriate for the simultaneous assay of all antiarrhythmics in this study. A gradient system was therefore investigated.

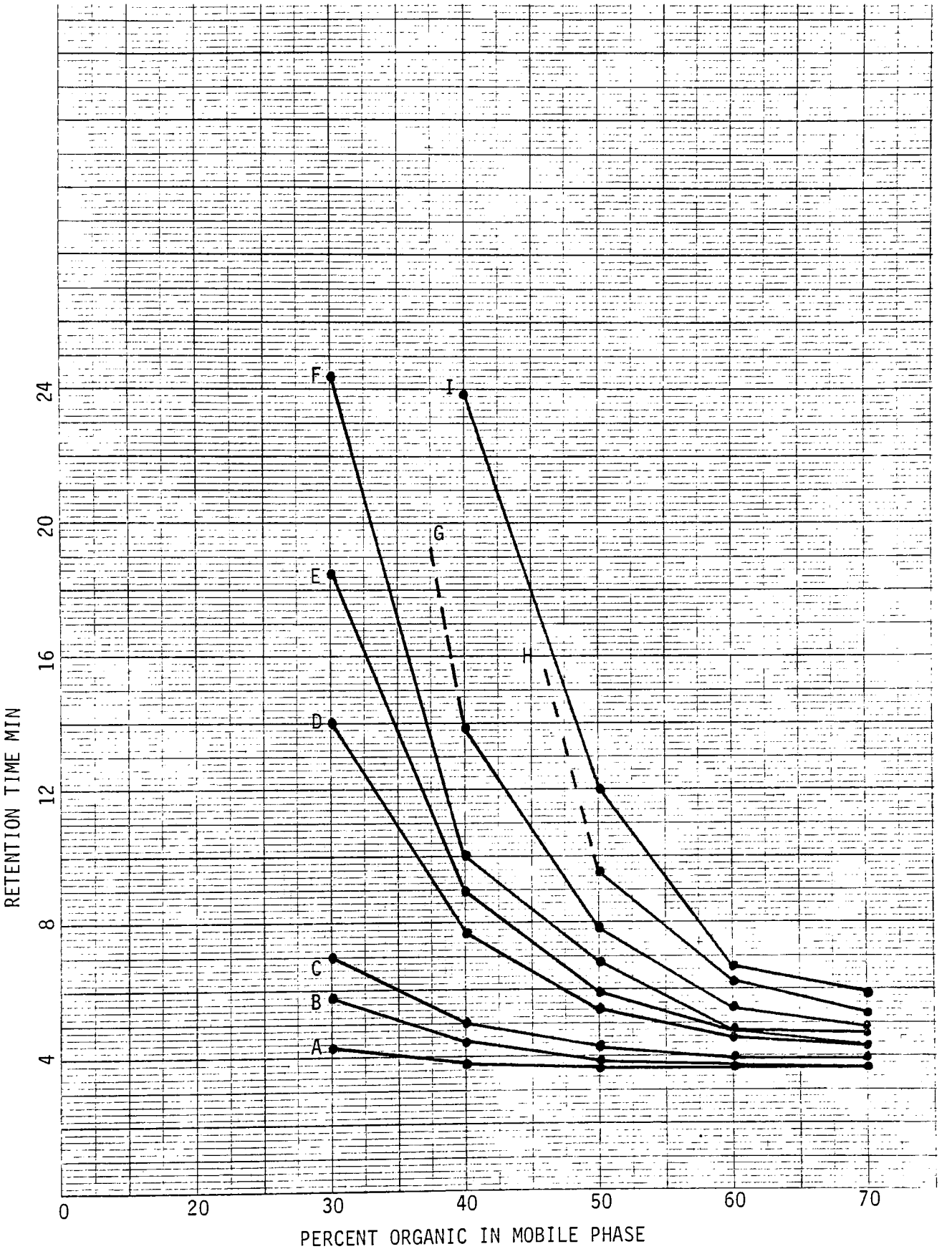

Fig. 24. Drug elution using isocratic analysis. Elution of assay components using mobile phase of fixed organic/phosphate composition throughout the run. A dashed line indicates that the component did not elute from the chromatograph at the indicated isocratic condition. Components are: A, procainamide; B, NAPA; C, NPP; D, NDAD; E, 4-OH propl; F, disop; G, quin; H, dihyq; I, propl.

Gradient Development

Several gradient programs were investigated to determine the best conditions for separation of all components in the assay. This was done by making adjustments in the length of time during which the mobile phase composition would change (i.e. the "slope" of the gradient), and by changing the organic/phosphate ratio at the beginning and end of the run.

In all runs, the mobile phase composition is constant for the initial 2 min. At 2 min the linear gradient, described below, begins. The gradient reaches a final mobile phase composition at the times shown and this composition is maintained until 25.5 min. The mobile phase then returns to its initial proportion to equilibrate the column for 5 min before the next run is initiated. The slope of the gradient is defined as the change in percent organic divided by the time, in minutes, during which the change takes place.

Experimental Linear Gradients

Gradient	Initial % Organic	Final % Organic	Gradient Termination	Slope % org/min
A	20	70	20 min	2.78
B	20	70	15 min	3.85
C	20	80	20 min	3.33
D	20	70	23 min	2.38
E	30	70	20 min	2.22
F	30	70	23 min	1.90

The slope of the gradient and initial percent organic are critical for adequate separation of all drugs. Steep slopes, such as 3.85% org/min in gradient B, do not resolve disopyramide and quinidine. Gradients using a higher initial percent organic, 30% in gradient E and gradient F, fail to adequately separate procainamide and NAPA. Gradients with intermediate slopes, using a 20% organic such as 2.78% org/min (gradient A), 3.33% org/min (gradient C), and 2.83% org/min (gradient D) provide the best resolution. Except for lidocaine and NDAD, all drugs are separated by at least 1.0 min. Gradient A resolved lidocaine and NDAD better than either gradients C or D and was therefore adopted as the gradient of choice. Gradients are presented in Figures 25-30.

METHOD OPTIMIZATION

After the mobile phase conditions had been decided upon, refinements

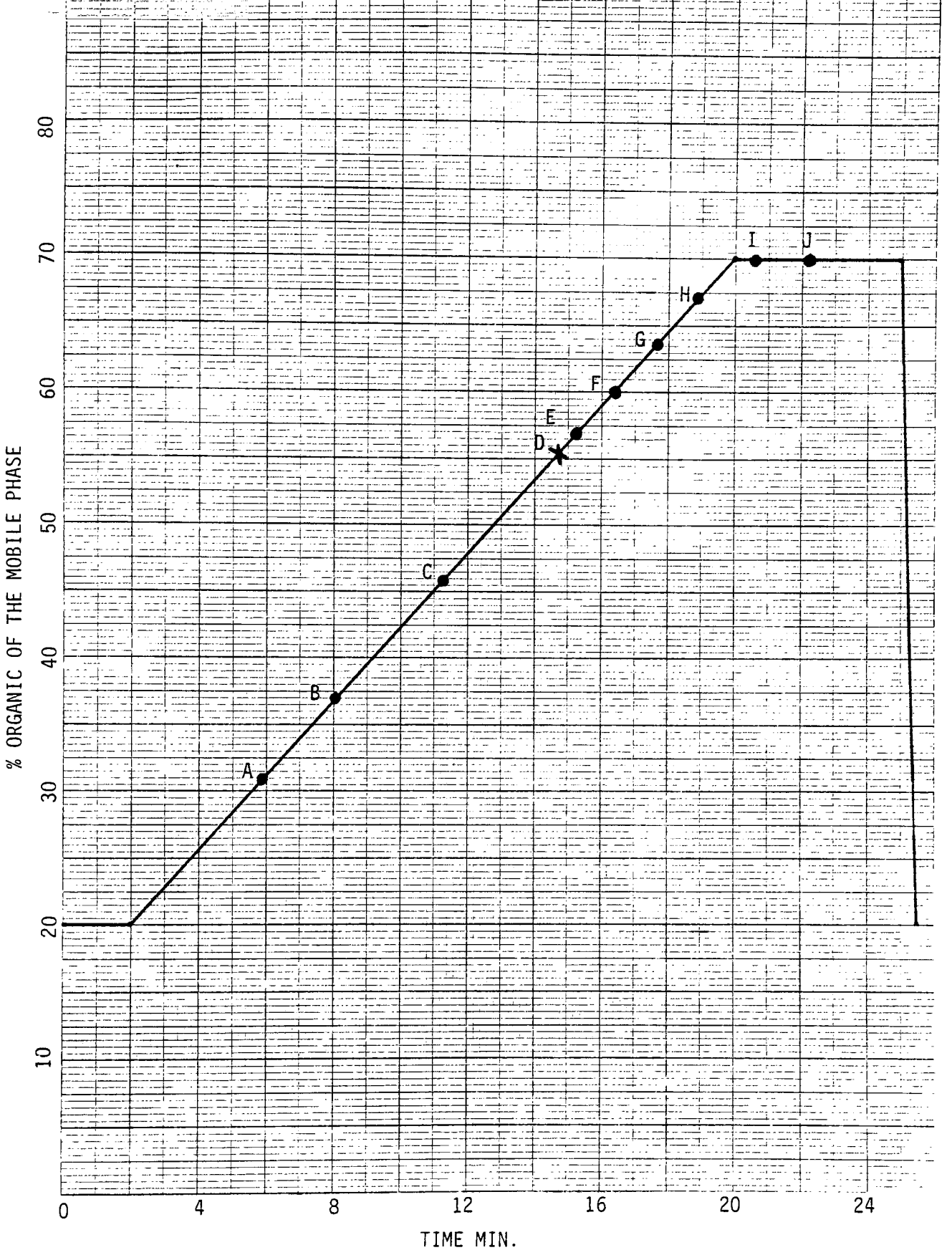

Fig. 25. Gradient elution. Change in percent organic of the mobile phase with time, during the run. Gradient A: initial % organic 20, final % organic 70, gradient termination 20 min, slope 2.78 % organic/min. Components are: A, procainamide; B, NAPA; C, NPP; D, lidoc; E, NDAD; F, 4-OH propl; G, disop; H, quin; I, dihyq; and J, propl.

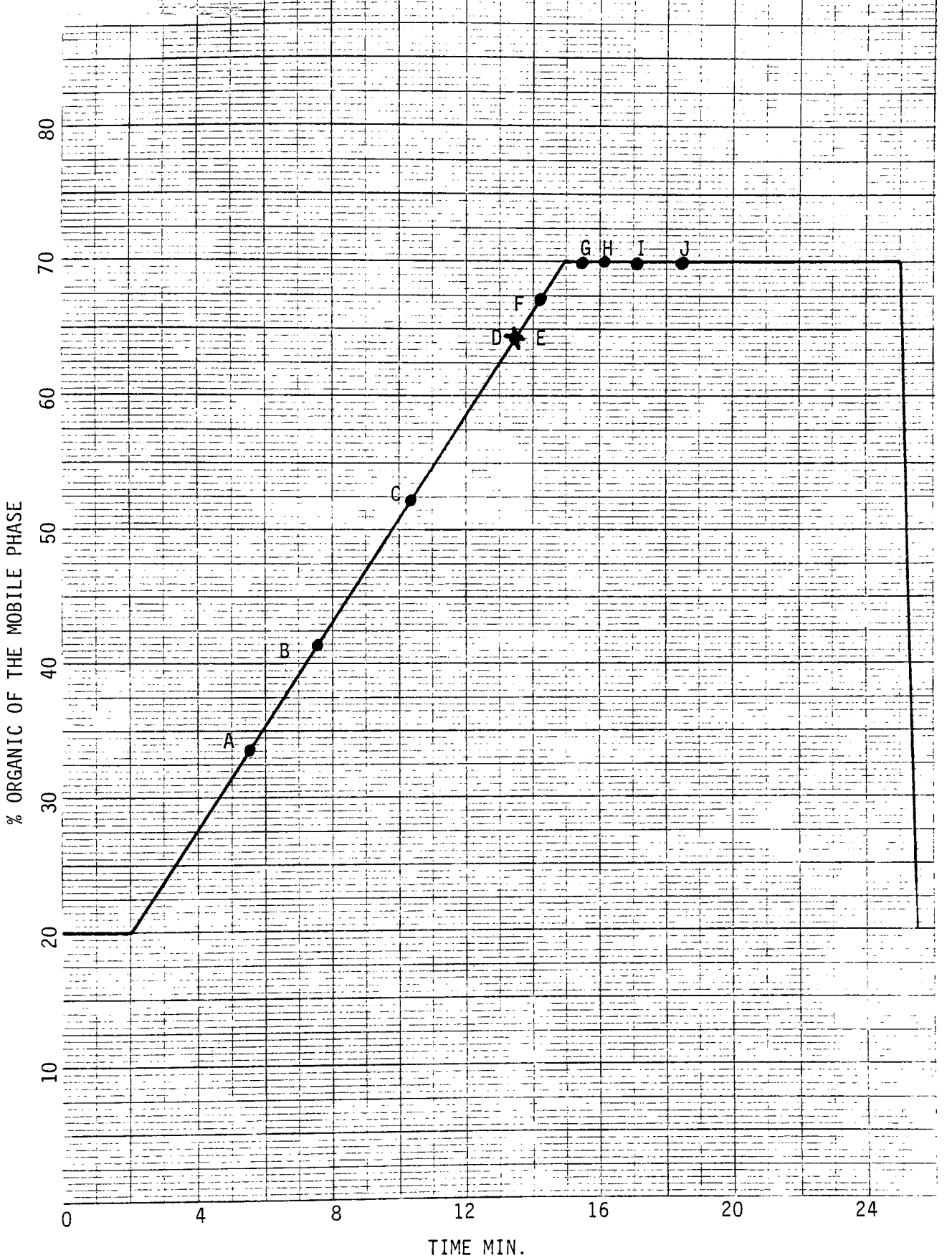

Fig. 26. Gradient elution. Change in percent organic of the mobile phase with time, during the run. Gradient B: Initial % organic 20, final % organic 70, gradient termination 15 min, slope 3.85 % org/min. Components are: A, procainamide; B, NAPA; C, NPP; D, lidoc and E, NDAD (coeluting); F, 4-OH propl; G, disop; H, quin; I, dihyq; and J, propl.

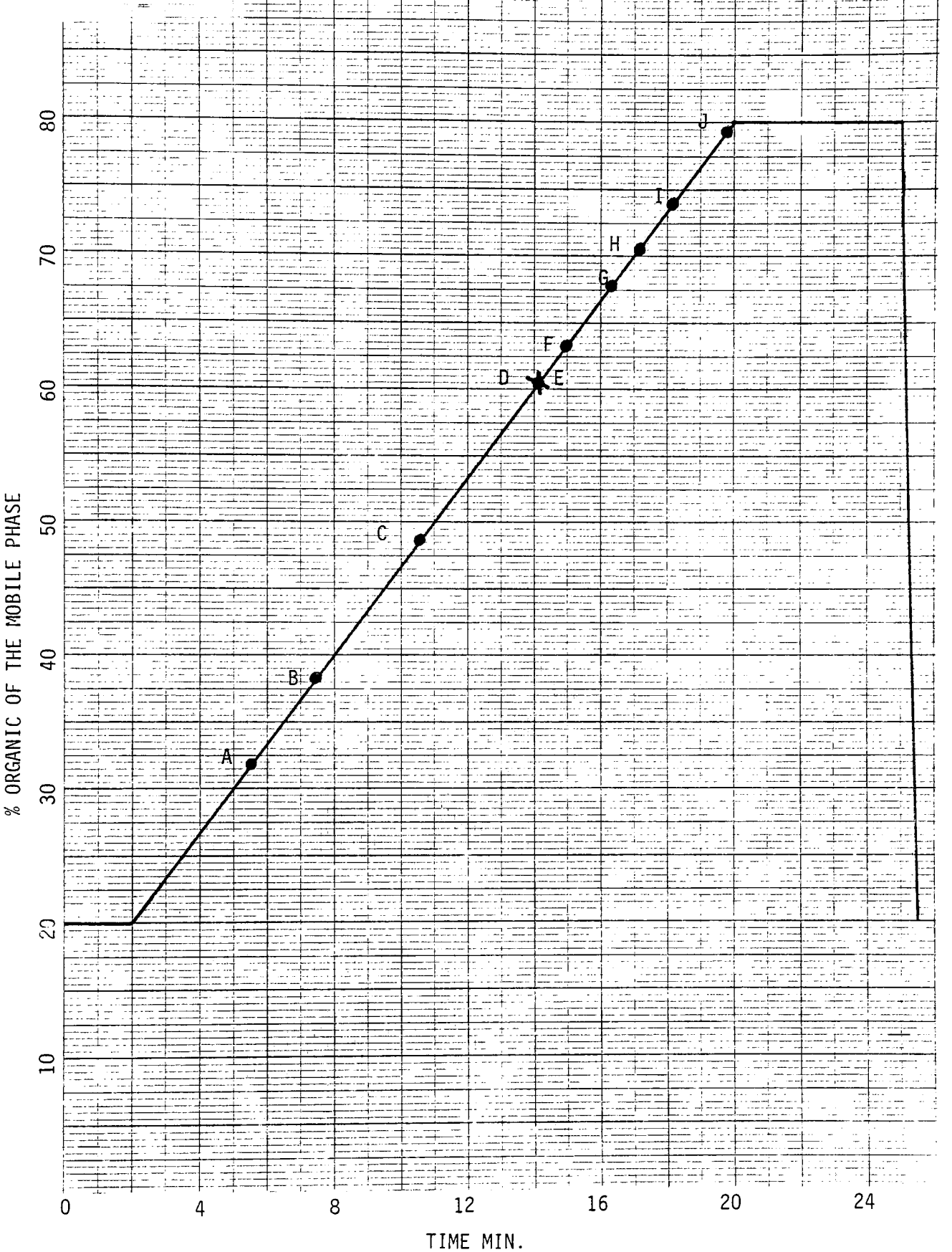

Fig. 27. Gradient elution. Change in percent organic of the mobile phase with time, during the run. Gradient C: Initial % organic 20, final % organic 80, gradient termination 20 min, slope 3.33 % org/min. Components are: A, procainamide; B, NAPA; C, NPP; D, lidoc and E, NDAD (coeluting); F, 4-OH propl; G, disop; H, quin; I, dihyq; and J, propl.

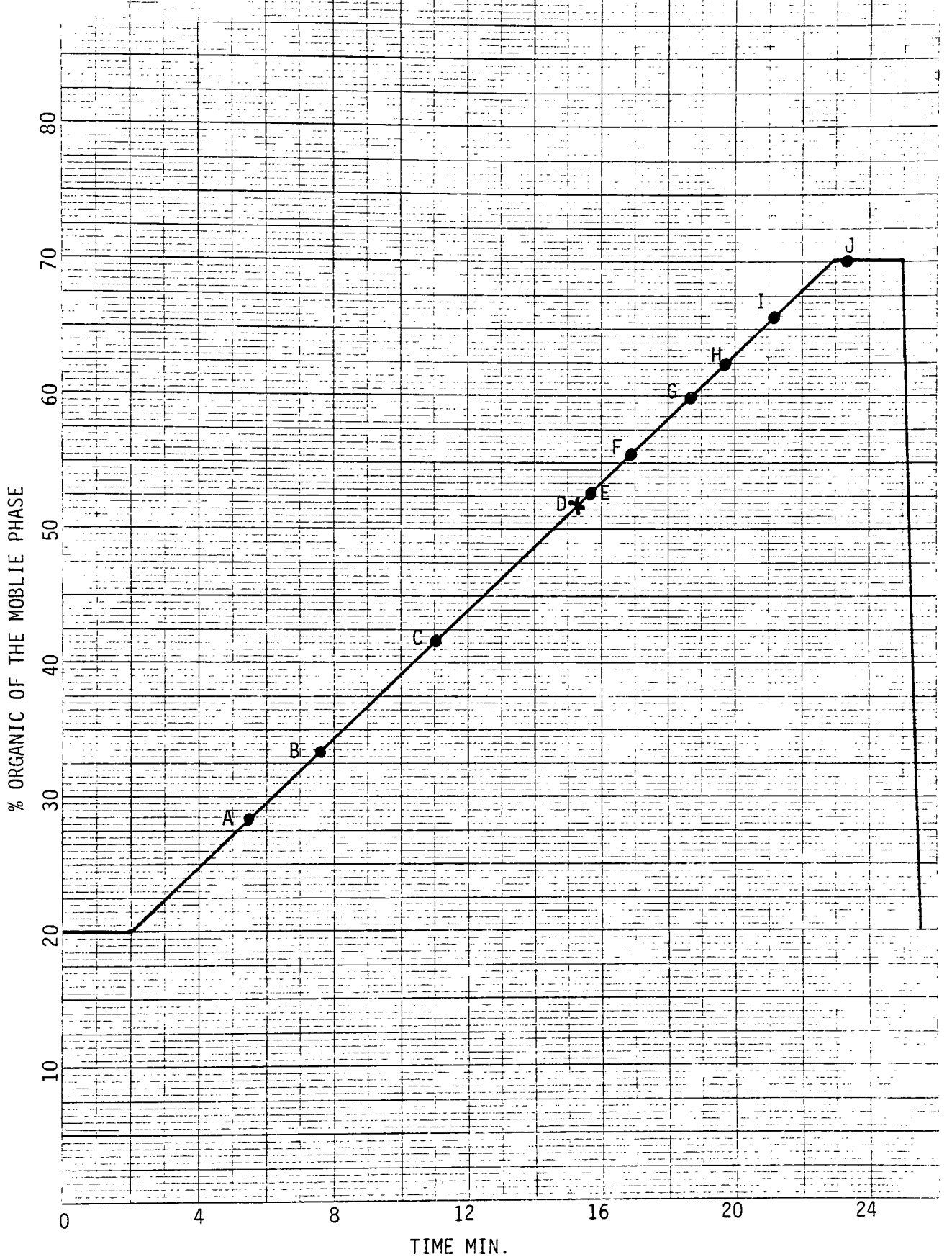

Fig. 28. Gradient elution. Change in percent organic of the mobile phase with time, during the run. Gradient D: Initial % organic 20, final % organic 70, gradient termination 23 min, slope 2.38 % organic/min. Components are: A, procainamide; B, NAPA; C, NPP; D, lidoc; E, NDAD; F, 4-OH propl; G, disop; H, quin; I, dihyq; and J, propl.

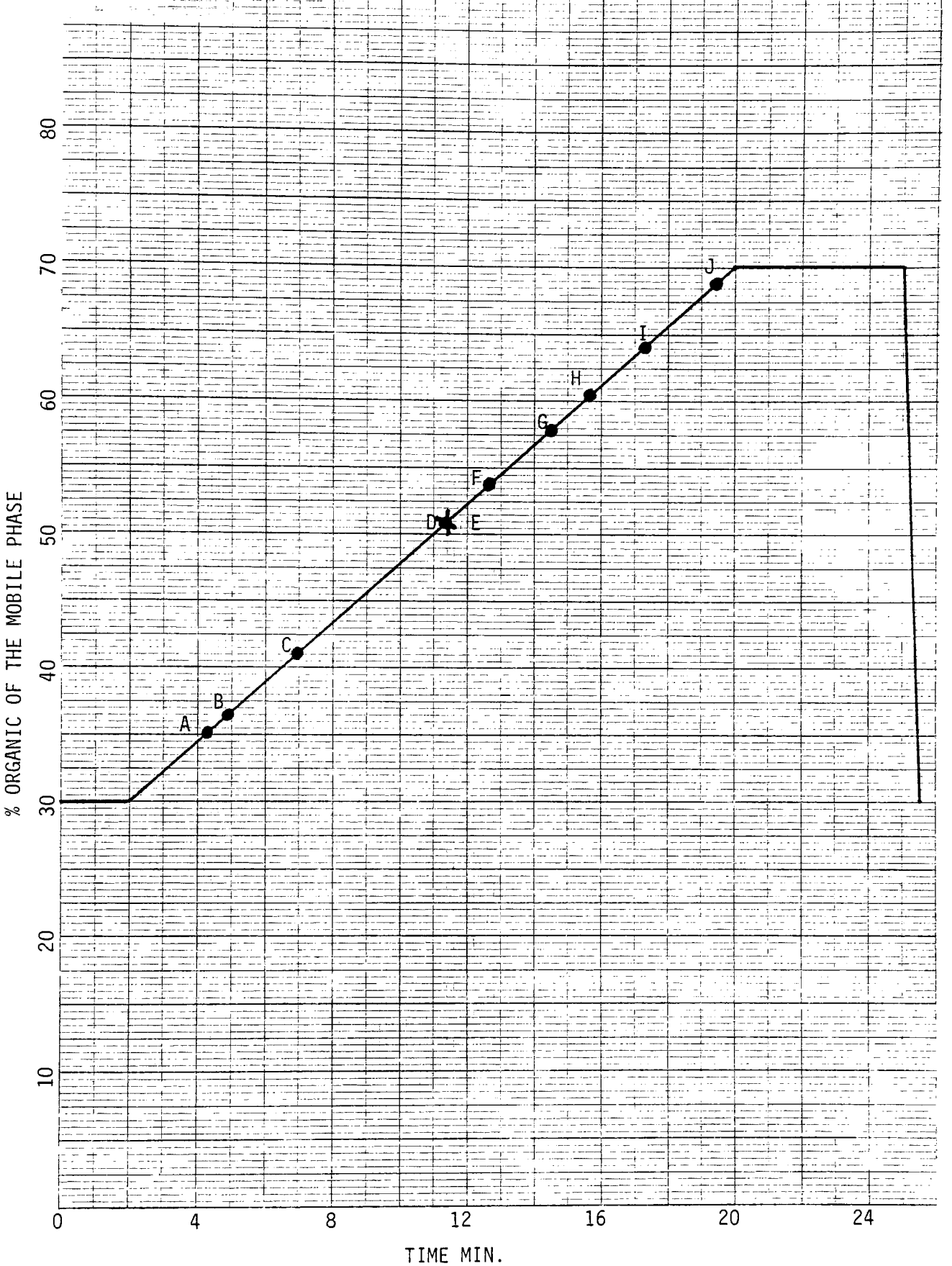

Fig. 29. Gradient elution. Change in percent organic of the mobile phase with time, during the run. Gradient E: Initial % organic 30, final % organic 70, gradient termination 20 min, slope 2.22 % org/min. Components are: A, procainamide; B, NAPA; C, NPP; D, lidoc and E, NDAD (coeluting); F, 4-OH propl; G, disop; H, quin; I, dihyq; and J, propl.

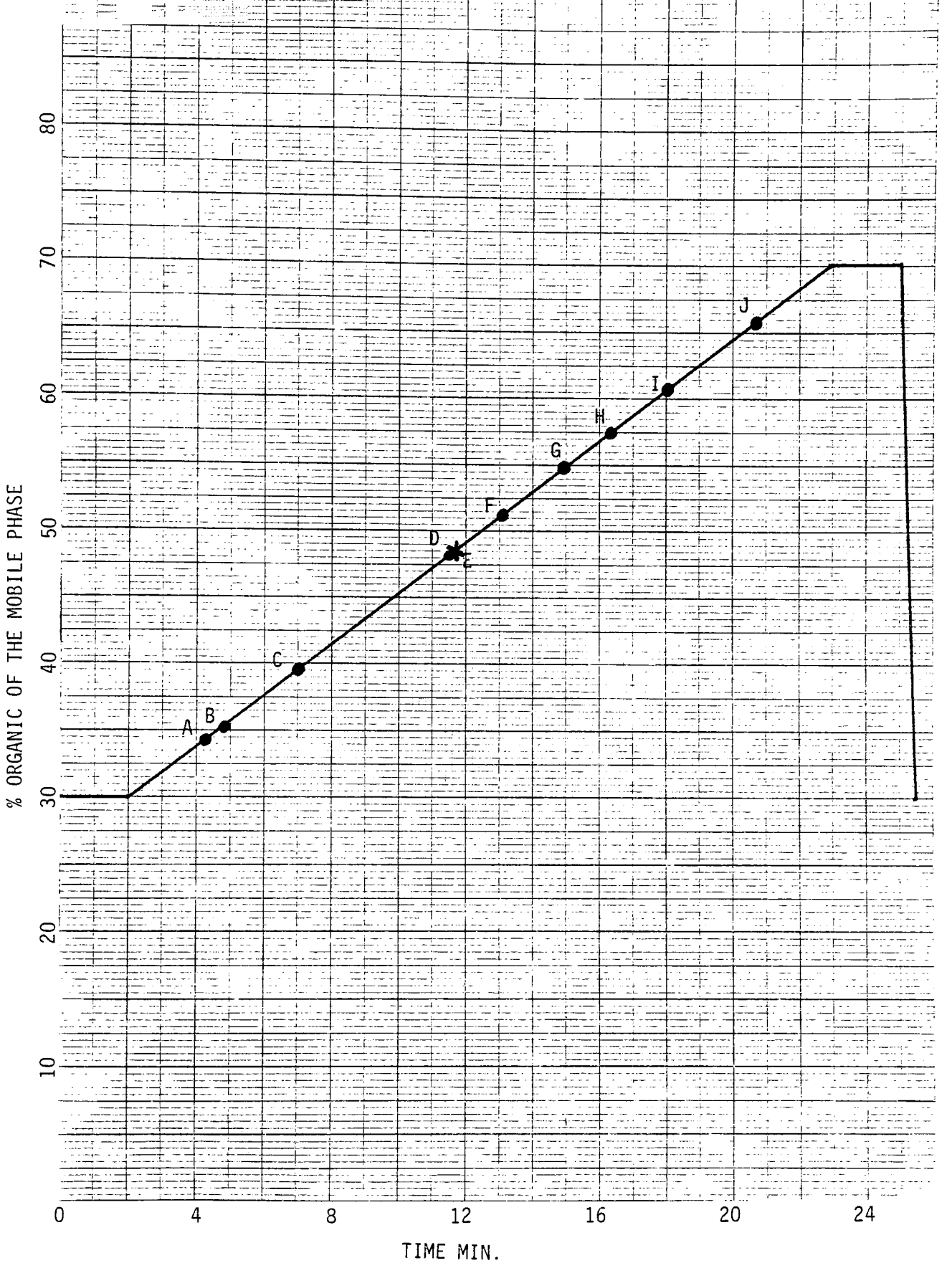

Fig. 30. Gradient elution. Change in percent organic of the mobile phase with time, during the run. Gradient F: Initial % organic 30, final % organic 70, gradient termination 23 min, slope 1.90 % org/min. Components are: A, procainamide; B, NAPA; C, NPP; D, NDAD; E, lidoc; F, 4-OH propl; G, disop; H, quin; I, dihyq; and J, propl.

were made in the instrument settings to select a detector wavelength where propranolols absorption would be maximum. This was necessary because of the drug's low therapeutic serum level, (20 - 100 µg/ml). Ultraviolet absorption scans were performed. The necessary specimen and injection volumes to be used in the assay were also determined with the propranolol sensitivity requirements in mind.

Wavelength For Analysis

All drugs were prepared in mobile phase at a concentration of 5.0 mg/L. Each was then scanned in a Perkin-Elmer 559 UV/VIS spectrophotometer from 500 nm to 190 nm. The liquid chromatograph is also capable of scanning but the time required is considerably longer than with the Perkin-Elmer. A liquid chromatographic scan of propranolol (Figure 31) is included for illustrative purposes.

The wavelength for analysis was chosen at propranolol's absorbance maximum of 212 nm. All other drugs have sufficient absorbance at this wavelength to allow accurate quantitation. A reference wavelength of 430 nm was selected because none of the drugs absorb at this wavelength. The data in Table 20 point out the advantage of a variable wavelength detector. Propranolol and lidocaine have over a 20 fold increase in absorptivity at 212 nm when compared to 254 nm. Seven of the other components have at least a two fold increase in absorptivity at the lower wavelength.

Assay Volumes

The assay volumes were selected to maximize the peak area of propranolol and thereby enhance its analytical sensitivity. The volume of mobile phase used for reconstitution after extraction was 150 µl. An injection volume of 75 µl was used. This volume allowed 50% of all extracted material to be injected into the chromatograph. This percentage is the maximum possible amount with the available vials and injection system.

The Clin-Elute CE-1003 column can handle a maximum of 3.0 ml of aqueous material without overloading the column matrix (19). By using 2.5 ml of serum and 200 µl of Na_2CO_3 in the extraction procedure, 2.7 ml of aqueous material is placed onto the column. The volumes selected for use in the assay protocol maximize the amount of drug injected into the chromatograph. This should allow quantitation of

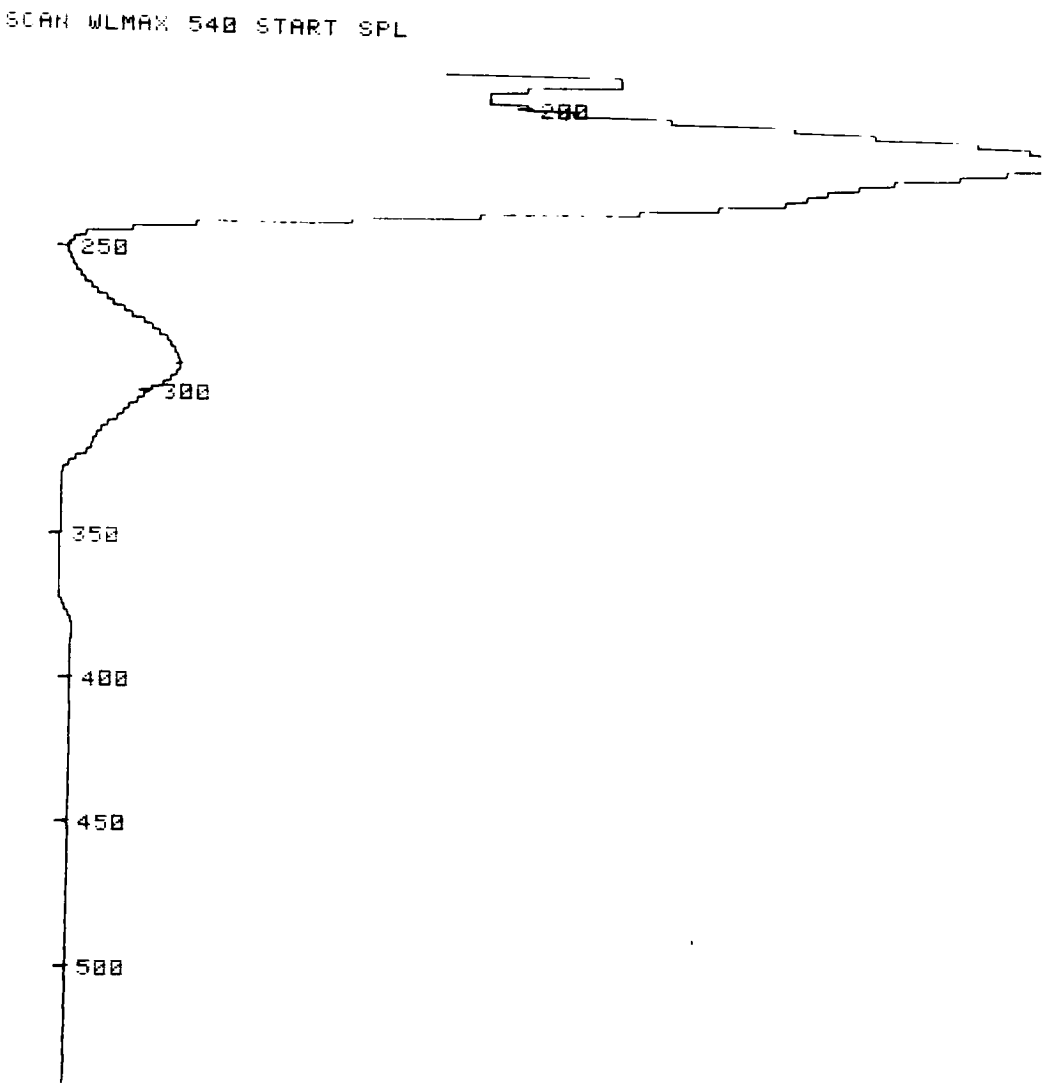

Fig. 31. HPLC ultraviolet absorbance scan of propranolol from 190 nm to 540 nm. Absorbance readings are taked every 2 nm. Wavelength (nm) is printed every 50 nm.

TABLE 20. ULTRAVIOLET ABSORBANCE SCANS AT SEVERAL WAVELENGTHS

	Absorbance[a]				
Drug[b]	205 nm	210 nm	212 nm	220 nm	254 nm
Procainamide	.55	.46	.45	.27	.23
NAPA	.61	.47	.40	.25	.35
NPP	.54	.44	.37	.26	.30
NDAD	.43	.32	.28	.20	.09
Disopyramide	.45	.32	.28	.16	.07
Quinidine	.74	.68	.61	.50	.35
Dihydroquinidine	.61	.56	.52	.41	.23
Propranolol	.75	.88	.88	.75	.04
Lidocaine	>1.0	>1.0	>1.0	.60	.05

[a] Instrument sensitivity 1.0 AUFS.

[b] Concentration (5 mg/L) in mobile phase.

propranolol in the therapeutic range of 20 - 100 µg/L.

Internal Standard Selection

Procaine, p-chlorodisopyramide, and N-propionylprocainamide were evaluated as suitable internal standards in the assay. Procaine was rejected as an internal standard because of its tendency to "tail" under the chromatographic conditions of the assay. P-chlorodisopyramide was also rejected. It eluted very late into the run (20 min) and had only a 74% extraction efficiency. It was also found to coelute with carbamazepine and potentially interfere with accurate quantification of propranolol (see Interference Study Table 9).

N-propionylprocainamide proved to be a suitable internal standard. It elutes about midway into the run and has an 84% extraction efficiency. None of the forty-two drugs tested in the interference study elute within 2 min of NPP. NPP was adopted as the internal standard for the assay protocol.

The amount of N-propionylprocainamide was selected in order to produce a peak area ratio of drug (or metabolite)/internal standard as near to unity as possible when the drug is in the therapeutic range. The variation of absorptivities at 212 nm, and the expected amount of material extracted from serum for each analyte, produced a large range in the ratios. The area ratio at the mean concentration of the run-to-run precision study is shown in Table 6. Even at the small peak area ratio for propranolol, the microprocessor is able to compute the area precisely enough to achieve a relative standard deviation of 5.1%.

DRUG EXTRACTION

Several organic solvents were tested for their ability to extract the drugs of interest from serum. Once extracted, the chromatographic conditions developed previously were used to quantify the drugs. In the first experiment, drugs were isolated from serum by manual liquid-liquid extraction. In the second part of the study, the drugs were extracted with the use of the Clin-Elute CE-1003 columns.

Manual Extraction

A control of all antiarrhythmic drugs, at their therapeutic concentrations was prepared by spiking pooled serum that was known to be drug free. The control was added to test tubes, followed by 1.0 mol/L Na_2CO_3 and the appropriate organic solvent. The Na_2CO_3 is necessary

to adjust the serum to a high pH (approximately 10) where the basic drugs, existing in an unionized hydrophobic form, can be easily extracted into the nonpolar solvent. The tubes were vortexed and centrifuged. The organic layer was then transferred to a new test tube to evaporate the organic solvent. Each residue was reconstituted with 150 µl of mobile phase, vortexed, and removed to a microvial for chromatographic analysis. Solvents of various polarities were selected to test the drugs extractions (acetonitrile, chloroform, methylene chloride, hexane, heptane, N-butyl chloride, ethyl acetate, isopropanol, and toluene). Each of these were also tested with the addition of 2% isoamyl alcohol.

Manual extraction proved to be a poor technique for the isolation of the drugs from serum. Some extraction tubes formed emulsions. Others produced a white turbid solution upon addition of the mobile phase. Long periods of time (30 min) were also required for evaporation of the extraction solvents. No useful information was gained from the manual extraction experiment.

Clin-Elute Extraction

Two types of Clin-Elute extractions were performed. The first employed only a single extraction step. The second utilized an acid back-extraction to transfer the basic drugs from the organic phase into a microvolume of aqueous HCl.

Single Extraction

Clin-Elute CE-1003 columns were prepared by adding 200 µl of 1.0 mol/L Na_2CO_3 and 50 µl of 0.2 mg/L N-propionylprocainamide to each column. Frozen serum controls of the antiarrhythmic drugs were thawed and 2.5 ml were then applied to appropriately labeled columns placed over 16x150 mm test tubes. Three additions of 4 ml of organic solvent were added to the columns. Each eluted solvent was evaporated to dryness at 50°C. The residues were redissolved in 150 µl of mobile phase and 75 µl of this solution was to be injected into the chromatograph. The solvents tested were acetonitrile, chloloform, methylene chloride, hexane, heptane, N-butylchloride, ethyl acetate, isopropanol, and toluene. Each of these solvents was also tested with the addition of 2% isoamyl alcohol v/v to reduce nonspecific adsorption to glass (20).

Isopropanol and acetonitrile (with of without isoamyl alcohol) would not pass through the column. The mobile phase reconstitution of the residues from the other solvent extractions produced milky suspensions.

To prevent possible damage to the system, the suspensions were not injected. Neutral extracting serum components may have been responsible for the suspensions. No useful extraction information was gained in this experiment.

Dual Extraction

Clin-Elute CE-1003 columns were prepared for extraction of basic drugs by the addition of 200 µl of 1.0 mol/L Na_2CO_3 and 50 µl of 0.2 mg/L N-propionylprocainamide to each column. The eluent tube placed under each column had 200 µl of 0.1 N HCl added to it. Frozen controls of antiarrhythmic drug in serum were thawed and 2.5 ml were applied to appropriately labeled Clin-Elute columns. Three addtions of 4 ml of organic solvent were then added to each column. The eluent tubes were each vortexed for 45 s to back-extract the basic drugs into the HCl. Each tube was centrifuged for 10 min, the organic layer was aspirated and discarded, and the aqueous HCl phase was dried under N_2, after 300 µl of methanol had been added. The residue in each tube was then reconstituted with 150 µl of mobile phase and 75 µl of this solution was injected into the chromatograph. The solvents tested were: methylene chloride, chloroform, ethyl acetate, toluene, hexane, heptane, N-butylchloride, methylene chloride:isopropanol (90:10), and methylene chloride:hexane (50:50). All of the above solvents and mixtures were also tested with the addition of 2% isoamyl alcohol.

Residues from methylene chloride:isopropanol extractions formed slightly cloudy suspensions upon addition of mobile phase. All other extractions were clear and colorless.

Solvents and mixtures which had densities less than the 0.1 N HCl were easy to work with because the HCl would remain as an easily discernible phase at the bottom of the test tube thus making aspiration of the organic a simple procedure. Solvents that were more dense than 0.1 N HCl proved to be undesirable because the HCl remained on top of the organic phase which made aspiration difficult. It was not possible to aspirate the organic phase from extractions that had solvent densities near 1.00 gm/cc because no separation of phases occurred after centrifugation (e.g. methylene chloride:hexane). Solvents with densities of less than 0.99 gm/cc should be used to avoid these aspiration problems.

As shown in Table 21, the more polar drugs, such as procainamide

TABLE 21. CLIN-ELUTE EXTRACTION EFFICIENCIES. PERCENT RECOVERY OF ASSAY DRUGS USING THE ESTABLISHED DXTRACTION PROTOCOL WITH VARIOUS ORGANIC SOLVENTS.

Solvent	PA	NAPA	NPP	NDAD	DISOP	QUIN	DIHYQ	PROPL
Ethyl acetate	112	117	98	109	105	102	114	26
Ethyl acetate:IAA[a]	113	119	97	105	107	104	114	16
Toluene	8	<1	2	1	66	90	74	115
Toluene:IAA	53	18	50	B4	106	106	112	100
Hexane	<1	<1	<1	<1	<1	<1	<1	62
Hexane:IAA	<1	<1	<1	<1	88	104	109	108
Heptane	<1	<1	<1	<1	<1	<1	<1	<1
Heptane:IAA	<1	<1	<1	<1	86	102	106	107
Butyl chloride	14	<1	5	<1	74	78	67	101
Butyl chloride:IAA	39	1	49	49	90	86	85	76
Methylene chloride:IPA[b]	114	112	75	84	18	49	37	<1
Methylene chloride:IPA:IAA[c]	112	104	66	78	16	38	27	<1
Methylene chloride	107	101	92	70	66	91	89	56
Methylene chloride:IAA	114	113	91	103	56	94	78	22
Chloroform	112	110	94	97	60	90	80	67
Chloroform:IAA	105	107	85	93	44	66	52	19
Methylene chloride:hexane[d]	-	-	-	-	-	-	-	-
Assay protocol solvent[e]	96	73	85	102	103	102	105	70

a IAA-isoamyl alcohol (ratio of solvent:IAA is 98:2).

b IPA-isopropyl alcohol (ratio of methylene chloride:IPA is 90:10)

c Ratio of methylene chloride:IPA:IAA is 88:10:2)

d Not assayed because phase separation did not occur.

e Methylene chloride:hexane:IAA 49:49:2.

and NAPA, extract well into the slightly nonpolar solvents, such as chloroform. Nonpolar drugs, like propranolol, extract best into very nonpolar solvents, such as toluene or hexane. The solvent system adopted, methylene chloride:hexane:isoamyl alcohol (49:49:2), takes advantage of the good recovery of propranolol by hexane-isoamyl alcohol and the good recovery of the other drugs by methylene chloride. In addition, the mixture has a density of 0.989 gm/cc, which ensures that the HCl will remain on the bottom of the tube after centrifugation.

CONCLUSION

We have developed a high performance liquid chromatographic assay that is of adequate sensitivity and precision for clinical use. The extraction and resolution of both drugs and metabolites of a "family" of pharmacological agents (cardioactive drugs) from spiked sera, allows the laboratory to use a standard procedure regardless of which drug or drugs are requested for analysis. The 30 min chromatographic run is not a major drawback because of the autosampling feature of the chromatograph that allows the assay to be performed (after extraction) without operator intervention.

The assay has only a limited number of interferences because most acidic and neutral drugs are removed by the extraction procedure. Although carbamazepine and glutethimide may interfere with the quantification of propranolol, they can be removed by additional organic extractions if the laboratory is made aware of their presence. Quinine and lidocaine cannot be eliminated as interfering agents by the same technique due to their basic character. Because it is impossible to analytically test all drugs and metabolites for interference, we are closely scrutinizing all patient chromatograms for unidentified peaks.

This specific and accurate liquid chromatographic assay does not suffer from the many interferences found in manual fluorometric and colorimetric methods. HPLC compares favorably with enzumeimmunoassay in its ability to accurately quantitate the drugs of interest. It also provides a means of monitoring some major metabolites which, with increased knowledge of drug action, may be clinically useful.

The extensive work performed on the development of the chromatographic conditions will be valuable in the implementation of future assays on this instrument. Several drugs which were tested as interferences are being considered for assay, utilizing the established chromatographic conditions.

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